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Hyeung-rak Kim

Louisiana State University and Agricultural & Mechanical College

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**Characterization and potential utilization of proteases from the
hepatopancreas of crawfish, *Procambarus clarkii***

Kim, Hyeung-Rak, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1991

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

**CHARACTERIZATION AND POTENTIAL UTILIZATION OF PROTEASES
FROM THE HEPATOPANCREAS OF CRAWFISH, *Procambarus clarkii***

A Dissertation

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy**

in

The Department of Food Science

by

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December, 1991**

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LIST OF ABBREVIATIONS

ATEE	Acetyl-L-tyrosine ethyl ester
BAEE	Benzoyl-L-arginine ethyl ester
BAPNA	Benzoyl-D,L-arginine-p-nitroanilide
BTEE	Benzoyl-L-tyrosine ethyl ester
D.W.	Distilled water
DEAE	Diethylaminoethyl
DFP	Diisopropyl fluorophosphate
Disc-PAGE	Discontinuous polyacrylamide gel electrophoresis
DTT	Dithiothreitol
E _a	Activation energy
EDTA	Ethylenediaminetetraacetate
eV	Electron voltage
GC/MS	Combined GC and MS
GC	Gas chromatography or Gas chromatograph
GPNA	N-p-Glutaryl-L-phenylalanine p-nitroanilide
HPLC	High performance (or pressure) liquid chromatograph
K _m '	Apparent Michaelis-Menten constant
m/z	Mass-to-charge ratio
MS	Mass spectrometry
MSD	Mass selective detector
p-CMB	p-Chloromercuribenzoate
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethanesulfonylfluoride
RI	Retention index or Retention indices

SBTI	Soybean trypsin inhibitor
SDE	Simultaneous steam distillation/solvent extraction
SDS	Sodium dodecyl sulfate or sodium lauryl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TAME	N-p-Tosyl-L-arginine methyl ester
TCA	Trichloroacetic acid
TES	N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid
TLCK	N-p-Tosyl-L-lysine chloromethyl ketone
TPCK	N-p-Tosyl-L-phenylalanine chloromethyl ketone
Tris	Tris[hydroxymethyl]aminomethane
V_{\max}	Substrate turnover number
V_{\max}/K_m'	Physiological efficiency

ABSTRACT

Four electrophoretically homogenous anionic trypsins, tentatively named trypsin A, B, C, and D, were isolated from crawfish hepatopancreas. Purity was increased 56, 100, 87, and 64-fold with approximately 2.4, 8.1, 5.1, and 3.0% yield for trypsin A, B, C, and D, respectively. The molecular weights of trypsin A, B, C, and D were estimated to be 23,800, 27,900, 24,800, and 31,400, respectively, using Sephacryl S-200 gel filtration. Isoelectric points and amino acid profiles of the trypsins were similar. Caseinolytic activity was consistently highest between pH 5.5 and 10.0 for all trypsins. Amidolytic and esterolytic activities were maximum in a narrower range, between pH 7.5 and 8.5. Trypsins were unstable at acidic pH. Thermal stabilities of the enzymes increased in the presence of casein. Trypsin C and D had higher thermal stability than trypsin A and B. Maximum activities of trypsin A, B, C, and D were achieved at concentrations of 0.5, 0.05, 0.025 and 0.1 mM Ca^{2+} ions, respectively. Sensitivity to inhibitors of these trypsins was similar to trypsin-like serine enzymes found in marine animals. Trypsin D had the highest substrate turnover number for caseinolytic reaction followed by trypsin A compared with other trypsins at physiological pH. This suggests that these two enzymes may play a primary role in the development of mushiness in tail meat. Crawfish trypsins had similar activation energies for the esterolytic reaction using

either N_α-p-tosyl-L-arginine methyl ester or benzoyl-L-arginine ethyl ester of approximately 6.4 to 9.0 Kcal/mole. The activation energies of the hydrolysis of benzoyl-D,L-arginine-p-nitroanilide ranged from 5.8 to 6.2 Kcal/mole and those for the hydrolysis of casein varied between 9.8 to 13.0 Kcal/mole. The optimum reaction conditions for the hydrolysis of crab waste protein by crawfish enzyme was established for enzyme concentration, pH and incubation time. One-hundred fifteen volatile flavor components were identified in enzyme treated crab waste and one-hundred sixteen in untreated crab waste. Volatile flavor compounds increased significantly in enzyme treated crab waste compared to untreated crab waste, however, high molecular weight aldehydes and aromatic hydrocarbons decreased in enzyme treated crab waste. 2-Ethyl-6-methylpyrazine and 2,3-dimethylpyrazine were among 12 identified pyrazine compounds with markedly higher concentrations in enzyme-treated sample.

CHAPTER I

INTRODUCTION

The Louisiana crawfish industry, with an annual production in excess of one hundred million pounds, provides crawfish tail meat, 15% by weight, to national and international foodservice markets year-round. The availability of crawfish tail meat has been successfully extended through frozen storage. However, freezing of whole crawfish has been discounted due to mushiness associated with the hepatopancreatic tissue. This phenomenon in fresh meat products has been an occasional and unpredictable problem. Mushy texture is mainly demonstrated after relatively short periods of iced storage of fresh meat, although deterioration during extended frozen storage also may occur (Godber et al., 1986).

The deterioration is presumed to be due to degradation of crawfish muscle proteins associated with hepatopancreatic proteases, since this phenomenon is most prominent in the anterior section of the tail. High activity of proteases in intestine of finfish accelerates autolytic degradation of abdominal tissues (Gildberg, 1978). Trypsin is quantitatively very important in the digestive system. Moreover, the participation of other proteases in muscle degradation is affected by the presence of trypsin due to its unique activation function of other enzymes. Marshall and co-workers

(1987) suggested the existence of proteolytic enzymes in the hepatopancreatic tissue of crawfish and indicated the importance of blanching for maintenance of textural quality. Heat labile proteolytic enzymes are involved in the development of mushiness in fresh crawfish meat, and loss of a desirable texture in fresh meat packed with adhering crawfish hepatopancreas was influenced by blanching time. Rowland et al. (1982) reported that mushiness in prawns is due to proteolytic enzymes diffusing from the digestive tracts, either from the hepatopancreas or from the gut running along the tail meat of the prawn.

Mushiness in muscle of prawn stored in ice may be caused by collagenase as measured by changes in soluble collagen during storage (Baranowski et al., 1984; Nip et al., 1985). Collagenases, assisted by a low level of proteolytic activity in hepatopancreas of prawn, Macrobrachium rosenbergii, may diffuse into the muscle tissue, causing the onset of tissue deterioration leading to mushiness (Lindner et al., 1988, 1989).

A variety of proteases have been isolated from the internal organs of fish and crustacea and characterized thoroughly as to their physicochemical and enzymatic properties. Digestive proteolytic enzymes have been studied in a wide range of decapod crustacea such as crab, lobster, shrimp, and crayfish. In crustacea, one of the major enzymes has been identified as trypsin-like and its physicochemical

and enzymatic properties have been characterized in crayfish (Zwilling and Tomasek, 1970; Zwilling et al., 1975; Zwilling and Neurath, 1981; Titani et al., 1983), crab (Muramatsu and Morita, 1981; Dendinger, 1987; Galgani and Nagayama, 1988; Dendinger and O'Connor, 1990), lobster (Galgani and Nagayama, 1987a), shrimp (Gates and Travis, 1969; Galgani et al., 1984) and Antarctic krill (Chen et al., 1978a; Osnes and Mohr, 1985a, b; Osnes et al., 1986). Other enzymes such as low molecular weight protease (Armstrong and DeVillez, 1978; Zwilling et al., 1981), collagenolytic serine proteinase (Eisen and Jeffery, 1969; Eisen et al., 1973; Grant and Eisen, 1980; Grant et al., 1983; Welgus et al., 1982; Welgus and Grant, 1983; Iida et al., 1991), and chymotrypsin-like enzyme (Kimoto et al., 1986; Jany, 1976; Kalac, 1978; Heu et al., 1991) also were demonstrated to play an important role in digestion and might be involved in postmortem muscle degradation. Although most characteristics of trypsin-like enzymes from crustacea are largely similar to vertebrate trypsins, some properties are markedly different. These differences include calcium ion requirements for enzyme catalysis and pH stability (Zwilling et al., 1969), extremely low isoelectric point (Zwilling et al., 1969; Kimoto et al., 1983), an extremely low level of basic amino acids (Zwilling and Neurath, 1981), and a wide range of molecular weights.

Among the proteases found in fish intestine, trypsin-like enzymes have been characterized as to physicochemical

properties (Prahl and Neurath, 1966; Camacho et al., 1970; Reeck and Neurath, 1972; Titani et al., 1975; de Haen et al., 1977; Cohen, et al., 1981a), enzymatic properties (Chen et al., 1978; Simpson and Haard, 1984a; Shin and Zall, 1987; Martinez et al., 1988; Guizani et al., 1991), inhibition properties (Grant and Eisen, 1980; Cohen et al., 1981b; Hjelmeland and Raa, 1982), and kinetic properties (Simpson and Haard, 1984b; Osnes and Mohr, 1985b; Stevens and McLeese, 1988). Those trypsins have physicochemical and enzymatic properties similar to mammalian trypsins.

Louisiana blue crab production is approximately 50 million pounds annually, of which approximately 75% is waste (Keithly, Jr., et al., 1988). After crab meat is picked, the residue of mostly shell, viscera, and legs has been traditionally discarded at sea or in landfills. However, such disposal is prohibited by the strict enforcement of pollution laws since 1985; alternate technologies, therefore, must be found to utilize or to dispose of the waste. In recent years, a series of studies has successfully demonstrated byproduct recovery including enzymes from fish waste (Shin and Zall, 1986; Reece, 1988; Olsen et al., 1990), astaxanthin pigments from crawfish waste (Chen and Meyers, 1982a, b; Meyers and Chen, 1985), and shrimp waste (Torrissen et al., 1981). Feed produced in part with dried byproduct from crawfish and shrimp increased natural pigmentation in salmonoids and egg (Lee, 1986). Chitin from crawfish shell has been demonstrated to

have commercial feasibility as a coagulant in recovery of organic compounds from seafood processing water (No and Meyers, 1989). Proteinaceous solids recovered from shrimp peeler effluent by HCl precipitation and centrifugation reduced total organic nitrogen and biochemical oxygen demand (Depaola et al., 1989). Also, HCl hydrolysate of crab waste had potential in various animal feeds, microbiological growth media and other food products, and provided alternate ways of dealing with crab waste disposal problems (Jaswal, 1990). Wash water from clam processing plants has been converted into a potentially marketable clam juice (Hood et al., 1976), a dehydrated clam flavor ingredient (Joh and Hood, 1979), a protein concentrate (Hang et al., 1980), and a natural flavoring agent (Burnette et al., 1983).

Recovery and utilization of blue crab waste that remains after picking meat is currently under investigation in the Dept. of Food Science, Louisiana State University, as a source of value-added seafood products. The hard-tissue material removed from soft tissue waste by a muscle/shell separator (Model 23-688, Stephan Paoli International, Rockford, IL) contains 12% nitrogen compounds and volatile flavor compounds that subjectively appear to be more abundant than in mince. Incorporation of enzymes from fish or crustacean viscera may improve the extraction of flavor compounds and increase the extractability of nitrogen compounds, mainly amino acids and peptides, by hydrolysis of protein. Also, this may provide a

potential use for crab waste as a crab flavoring agent.

The major objectives of the present investigation were to clarify the potential role of hepatopancreatic enzyme in the degradation of muscle proteins and potential as a recoverable byproduct for industrial application. Also, digestive proteolysis and distribution of proteolytic enzymes in the hepatopancreas of crawfish may provide understanding of crawfish digestive function and developmental evolution.

Based on the aforementioned, the specific objectives of the present research were:

- 1) Purification of trypsins from crawfish hepatopancreas.
- 2) Characterization of enzymatic properties of trypsins from crawfish hepatopancreas.
- 3) Characterization of kinetic and thermodynamic properties of trypsins from crawfish hepatopancreas.
- 4) Investigation of degradation of myofibrillar proteins from crawfish tail meat by trypsins.
- 5) Utilization of the aforementioned enzymes for extraction of nitrogen compounds and flavor compounds from crab waste.

CHAPTER II

REVIEW OF LITERATURE

1. Distribution of Trypsin

Trypsin and trypsin-like enzymes have been reviewed throughout the animal kingdom from invertebrates to mammals (Desnuelle, 1960; Walsh, 1970; Keil, 1971; Zwilling and Neurath, 1981). Trypsin is a pancreatic serine protease with substrate specificity based on positively charged lysine and arginine side chain in proteins or peptides (Brown and Wold, 1973; Krieger et al., 1974). Also, a serine and a histidine residue participate in the catalytic mechanism. The enzyme is synthesized as a zymogen type and stored in the zymogen granules. In higher animals, it is secreted into the intestine after a suitable stimulus, but in crustacea it is secreted into the cardia, a stomach-like organ.

Activation of trypsinogen to trypsin occurs by removal from the trypsinogen of a highly negatively charged hexapeptide at the N-terminal. Almost all proenzymes of pancreatic tissue are activated physiologically in the intestine by trypsin. Hence, understanding the properties of trypsin is very important in the digestive system and in degradation of muscle protein.

Trypsin has been isolated and characterized from higher vertebrates such as turkey (Ryan, 1965), sheep (Travis, 1968), bovine (Schroeder and Shaw, 1968), porcine (Walker and Keil,

1973), canine (Ohlsson and Tegner, 1973), rat (Vandermeers et al., 1973), human (Travis and Roberts, 1969; Figarella et al., 1975), elephant seal (Bricteux-Gregoire et al., 1974), whale (Bricteux-Gregoire et al., 1975), and moose (Stevenson and Voordouw, 1975). Also, trypsin has been isolated and characterized from teleost fish such as African lungfish (Reeck and Neurath, 1972), dogfish (Titani et al., 1975), sardine (Murakami and Noda, 1981), carp (Cohen et al., 1981a), capelin (Hjelmeland and Raa, 1982), catfish (Yoshinaka et al., 1983), Greenland cod (Simpson and Haard, 1984a), mackerel (Kim, 1986), skipjack (Pyeun et al., 1988), anchovy (Martinez et al., 1988), menhaden (Pyeun et al., 1990), Atlantic cod (Raae and Walther, 1989; Asgeirsson et al., 1989; Simpson et al., 1990), and Pacific salmon (Pivnenko et al., 1990).

Invertebrate organisms from which trypsin has been isolated and characterized include: crayfish (Pfleiderer et al., 1967), silkworm (Kafatos et al., 1967), earthworm (Bewley and Devillez, 1968), blackflies (Yang and Davies, 1968), white shrimp (Gates and Travis, 1969), starfish (Camacho et al., 1970; Winter and Neurath, 1970; Bundy and Gustafson, 1973; Kozlovskaya and Elyakova, 1974), crayfish Astacus leptodactylous (Zwilling and Tomasek, 1970; Zwilling and Neurath, 1981), Antarctic krill (Kimoto et al., 1983; Osnes and Mohr, 1985a), crab (Muramatsu and Morita, 1981), crayfish Procambarus clarkii (Kim et al., 1989a, b; Kim and Godber, 1990), Indian white prawn (Honjo et al., 1990), Atlantic blue

crab (Dendinger and O'Connor, 1990), and grass shrimp (Jiang et al., 1991).

2. Assay Methods for Trypsin Activity

Trypsin substrates can be described by the general formula, $R-CO-X$, where the confined specificity of trypsin is determined by the acyl moiety ($R-CO-$) and the type of bond cleaved is defined by the nature of X , such as peptide, ester or amide (Walsh, 1970). Preferred amino acid substrates can be described by the formula $R-Arg-X$ and $R-Lys-X$, where R is acetyl, benzoyl, carbobenzoxy, or tosyl residue; the group X released can be an ethyl or methyl ester as well as a nitroanilide, naphthylamine, thiobenzyl alcohol, or methylcoumarin group (Geiger and Fritz, 1981).

A. Proteolytic Activity

Protein-based assays are most generally applicable to all endopeptidases, including trypsin. Trypsin activity can be measured by degradation of denatured standard preparation of hemoglobin or casein (Anson, 1939; Laskowski, 1955). Trypsin liberates acid-soluble tyrosine- and tryptophan-containing fragments that can be quantitatively determined by the method of Folin and Ciocalteu (1927). This standard method using protein substrate has been modified by Barrett (1972) and Rick (1974).

B. Esterolytic Activity

The most widely used assays for tryptic activity are esterolytic activity toward benzoyl-L-arginine ethyl ester (BAEE) (Schwert and Takenaka, 1955), or p-tosyl-L-arginine methyl ester (TAME) (Hummel, 1959) by potentiometric or spectrophotometric procedure. A spectrophotometric method using TAME has advantages over BAEE in its greater sensitivity and greater selectivity (Hummel, 1959).

C. Amidolytic Activity

Amidase activity of trypsin is commonly determined using benzoyl-D,L-arginine p-nitroanilide (BAPNA) (Erlanger et al., 1961). Stewart (1973) has described a method for automated analyses of the tryptic activity using chromogenic substrates, such as BAPNA and N-glutaryl-L-phenylalanine p-nitroanilide (GPNA).

3. Purification of Trypsin

Different numbers of trypsin isoforms have been found for most animal species, with cationic forms predominating (Schroeder and Shaw, 1968; Keil, 1970). Anionic forms represent minor quantities and differ considerably from the cationic forms in a number of mammalia (Walsh, 1970). Nevertheless, marine vertebrate and invertebrate possess the anionic forms as the principal trypsin (Reeck et al., 1970; Overnell, 1973). Anionic forms have not been studied

extensively since methods previously developed for cationic enzymes for isolation and purification are not applicable to anionic enzymes.

Trypsin from fish intestine, especially crustacean intestine, is mainly anionic trypsin, extremely unstable and irreversibly inactivated in acidic pH range. Acid extraction has to be avoided and anionic exchange chromatography must be used instead of cationic exchange. Purification procedures for crayfish trypsin were established using anionic exchange chromatography and gel filtration by Zwilling et al. (1969) and Kim et al. (1989a). Since this method is very tedious, affinity chromatography such as benzamidine or soybean trypsin inhibitor coupled to matrix may be applicable to isolate crustacean trypsin. However, this enzyme-inhibitor complex cannot be dissociated at pH 3.0 or below, which differs from bovine trypsin. Therefore, a modified elution method using high pH of eluent or high concentration of competitive inhibitors, such as benzamidine at pH 8.0, is applicable to efficiently isolate crustacean trypsin (Zwilling and Neurath, 1981).

4. Properties of Trypsin

A. Molecular Weight

According to Keil (1971), trypsins have a M.W. ranging from 20,000 to 25,000. Trypsins in fish are similar to mammals

with respect to molecular weights as well as other enzymatic properties. In the intestine of fish, molecular weights (M.W.) around 20,000-25,000 have been reported for trypsins from sardine (Murakami and Noda, 1981), carp (Cohen et al., 1981a), capelin (Hjelmeland and Raa, 1982), catfish (Yoshinaka et al., 1983), Greenland cod (Simpson and Haard, 1984a), mackerel (Kim and Pyeun, 1986), skipjack (Pyeun et al., 1988), Atlantic cod (Asgeirsson et al., 1989; Simpson et al., 1990), and menhaden (Pyeun et al., 1990).

The M.W. of trypsin-like enzymes from crustacea vary widely, similar to those from fish intestine. A trypsin-like enzyme estimated to have a M.W. of 24,000 has been isolated from crayfish Astacus leptodactylous (Zwilling and Tomasek, 1970), white shrimp Penaeus setiferus (Gates and Travis, 1969) and Japanese spiny lobster Panulirus japonicus, (Galgani and Nagayama, 1987b). The M.W. of the trypsin-like enzymes obtained by gel filtration were reported to be 25,000 from lobster (Brockhoff et al., 1970) and shrimp (Galgani et al., 1984), and 29,000 from crab Eriocheir japonicus (Muramatsu and Morita, 1981). Also, slightly higher M.W. for trypsins from marine invertebrate has been reported, i.e., 30,000 to 31,000 from antarctic krill (Osnes and Mohr, 1985a) and 33,500 from crab Callinectes sapidus (Dendinger and O'Connor, 1990).

An extremely low M.W. (11,000-13,000) protease, which has similar enzymatic properties to trypsin, has been found in the digestive organ of decapods and might be a member of an

unknown family of proteases (Pfleiderer et al., 1967). This enzyme has not been reported in vertebrates. Such enzymes may act in a compensatory manner since there is an apparent absence of peptidic and chymotryptic endopeptidases in crustacean (Devillez, 1975). These enzymes have been observed in the digestive organs of crustacea such as lobster (Brockhoff et al., 1970; Galgani and Nagayama, 1987b), crab Carcinus meanas (Herbold et al., 1971), shrimp Penaeus spp (Galgani et al., 1984), and crayfish Orconectes virilis (Armstrong and Devillez, 1978).

B. Amino Acid Composition and Isoelectric Point

The amino acid compositions of trypsins from different species are well known; including humans (Travis and Robert, 1969) and bovine (Walsh and Neurath, 1964). The similarities in amino acid compositions among the trypsins reflect a considerable measure of homology in primary structure (Prahl and Neurath, 1966). The primary structure of bovine trypsinogen is a single polypeptide structure of 229 amino acid residues, crosslinked by 6 disulfide bonds (Hartley et al., 1965). The amino acid composition of crustacea has some marked differences from that of bovine trypsin (Bradshaw et al., 1970; Walsh and Neurath, 1964). Specifically, crustacean trypsin, like anionic trypsin from fish intestine, contains low levels of basic amino acid residues compared to mammalian trypsin.

The anionic nature of the trypsin-like enzymes might originate from the amino acid composition, which shows a very high proportion of acidic amino acid residues. Anionic trypsins seem to be quite common in marine organisms, and have been described in shrimp (Gates and Travis, 1969), starfish (Winter and Neurath, 1970; Kozlovskaya and Elyakova, 1974), crab (Muramatsu and Morita, 1981), capelin (Hjelmeland and Raa, 1982), catfish (Yoshinaka et al., 1984), krill (Osnes and Mohr, 1985b; Kimoto et al., 1983), anchovy (Martinez et al., 1988) and Pacific salmon (Pivnenko et al., 1990).

Trypsins from red swamp crayfish Procambarus clarkii as well as crayfish Astacus leptodactylous (Zwilling and Tomasek, 1970) and shrimp (Gates and Travis, 1969) also have a higher content of acidic amino acids than does bovine trypsin. Trypsins from both crayfish and shrimp had more than twice the number of acidic amino acids compared with bovine trypsin (Walsh and Neurath, 1964). The high ratio of acidic amino acid to basic amino acid residues supports the observation that these enzymes have low isoelectric points (Zwilling and Neurath, 1981).

Isoelectric points of trypsin-like enzymes from crustacean digestive enzyme have been reported to be very low, i.e., 4.0 for the Atlantic blue crab Callinectes sapidus (Dendinger and O'Connor, 1990), 3.8 for crayfish (Zwilling et al., 1969), 2.6 for Arctic krill (Kimoto et al., 1983), and less than 3.5 for Antarctic krill (Osnes and Mohr, 1985b).

This contrasts with bovine trypsin, which has a high isoelectric point between 9.3 and 10.1 (Walsh, 1970). Also the low molecular weight protease purified from crayfish Astacus fluviatilis is interesting because of its low isoelectric point as well as low molecular weight (Pfleiderer et al., 1967).

C. Stability

pH: Stability of enzyme at different pH changes with protonation or deprotonation of ionizing groups in the enzyme molecule and results in a protein conformation change. The state of ionization of the α -amino group appears to control the active conformation and substrate binding ability of the enzyme (Olafson and Smillie, 1975).

Studies of enzyme stability at different pH levels are performed to evaluate conditions for the most efficient practical application of enzyme storage. Bovine trypsin is stable at pH 3.0 and low temperature where it can be stored for weeks without loss of activity (Walsh, 1970). However, inactivation of bovine trypsin occurs at neutral pH due to autolysis (Zwilling et al., 1969). Porcine trypsin is more stable in the alkaline pH range than bovine trypsin because of structural differences (Vithayathill et al., 1961).

Trypsin from marine vertebrates and invertebrates is very stable for a long period at neutral pH, and CaCl₂ has no stabilizing or activating effect (Osnes and Mohr, 1985b). It

is irreversibly denatured at pH 3.0, which is opposite to that of bovine trypsin. The inactivation of trypsin from marine animal in an acidic pH may be a conformationally irreversible change, but the inactivation of bovine trypsin might be caused by autolysis.

Greater stability of crustacean trypsins could be ascribed to a fewer number of trypsin-labile bonds if they have an authentic specificity for peptide bonds involving the carboxyl group of lysine or arginine residues (Osnes and Mohr, 1985b). Also, the high content of acidic amino acids in the enzyme molecule might be distributed in the surface of the molecule, which causes the enzyme molecules to be repelled from each other. This repulsion among molecules may be a basis for stability against self degradation.

Three trypsins from krill, Euphausia superba, were inactivated in less than 1 hr at pH 4.5, whereas they were stable at pH 7.5 for 17 days without significant loss in activity. The enzymes were quite stable in alkaline media; activities decreased slowly at pH 9.6 (Osnes and Mohr, 1985b) as occurred for trypsin from shrimp (Gates and Travis, 1969).

Temperature: Enzymes are inactivated in media at high temperatures due to both the partial unfolding of the enzyme molecule and covalent alterations in the primary structure of the molecule (Tanford, 1968; Ahern and Klibanov, 1985). The mechanism for increasing thermal stability of proteins appears

to be due to strengthening of hydrophobic interactions in the interior of the protein molecule (Wedler and Hoffmann, 1974). Also, thermal stability is increased by a stabilizing helix rather than by sheet formation (Argos et al., 1979).

Higher numbers of intramolecular disulfide linkages contribute to higher thermostability of proteins (Komatsu and Feeney, 1970; Wedler and Hoffmann, 1974). Disulfide bonds can contribute stability under physiological conditions, and may stabilize a folded conformation that is no longer thermodynamically stable (Creighton, 1983). Simpson and Haard (1984b) suggested that lower resistance to thermal inactivation in cod trypsin may be caused by a lower number of maximal intramolecular disulfide bonds (4) compared to bovine trypsin, which has 6 disulfide bonds. However, Asgeirsson et al. (1989) indicated that structural destabilization may be due to differences in strength of weak intramolecular interactions rather than to fewer disulfide bonds.

Partially purified enzymes are more stable against thermal inactivation than are purified enzymes (Osnes and Mohr, 1985b). Hence, it can be assumed that when enzyme is present along with other proteins greater thermal stability occurs. Trypsin from Atlantic blue crab was stable from 30 to 50°C for 30 min, but activity was rapidly lost above 50°C (Dendinger and O'Connor, 1990). Tryptic activities from the hepatopancreas of jonah and rock crab were reduced 60% of original activities by incubation at 50°C for 20 min but the

enzymes were stable at 40°C for 20 min (Brun and Wojtowicz, 1976). Three alkaline proteinases from pyloric caeca of mackerel were stable up to 45°C for 5 min but most enzymatic activity was lost at 50°C (Kim and Pyeun, 1986). Uchida et al. (1984) reported that two anionic trypsins were stable at 35°C; activities decreased with increased temperature with ultimate inactivation at 55°C.

Ca²⁺ ion: The presence of calcium ions activates trypsinogen to trypsin and increases the thermal stability of the enzyme. This stabilizing effect is accompanied by a conformational change in the trypsin molecule, resulting in a more compact structure (Dehlquist et al., 1976; Hachimori et al., 1979). The protective effect of calcium ions is much more pronounced with bovine and ovine trypsin than with porcine trypsin (Buck et al., 1962).

Titani et al. (1975) reported that bovine and dogfish trypsin, but not porcine trypsin, bind calcium ions. The site of binding has not been established, but Stroud et al. (1971) have suggested that a combination of Asp₉₉, Asp₁₃₉, and Glu₆₅ may be effective in chelating calcium ions in the bovine enzyme.

Although calcium ions are activators for bovine trypsin activity, the enzyme undergoes rapid autolysis in the presence of Ca²⁺ ions, particularly in alkaline solutions (Zwilling et al., 1969). On the other hand, trypsin-like enzyme activity is inhibited by calcium ions in blue crab and in crayfish

(Zwilling et al., 1969). Calcium ions did not influence the tryptic activity of shrimp (Gates and Travis, 1969; Galgani et al., 1984) or spiny lobster (Galgani and Nagayama, 1987b).

D. Inhibition

Trypsin-like enzymes are generally characterized through inhibition by diisopropyl fluorophosphate (Desnuelle, 1960), phenylmethanesulfonyl fluoride (Fahrney and Gold, 1963), soybean trypsin inhibitor (Kakade et al., 1969), and amino acid derivatives of chloromethyl ketone (Schoellmann and Shaw, 1963). The essential structure of the active site in trypsin from fish or crustacean is the same throughout the vertebrate kingdom.

N-p-Tosyl-L-lysine chloromethyl ketone (TLCK) and benzamidine are well known as specific trypsin inhibitors. TLCK inactivates only trypsin-like enzyme by alkylation of the active-site histidine (Shaw et al., 1965; Shaw, 1980). It is known to make a covalent bond with the histidine of the catalytic portion and to block the substrate-binding part of the active center of the molecule (Severin and Tomasek, 1965). Although inhibition by TLCK is difficult to interpret appropriately because of possible side reactions with -SH groups, trypsins from mammalia to marine invertebrates are inhibited by this reagent (Zwilling and Neurath, 1981; Hjelmeland and Raa, 1982; Osnes and Mohr, 1985b; Galgani and Nagayama, 1987b; Martinez et al., 1988; Kim et al., 1989b;

Dendinger and O'Connor, 1990; Honjo et al., 1990; Pyeun et al., 1990).

Leupeptin and antipain, which have the argininal residue at their terminal carbon and inhibit trypsin and papain that cleave the carboxyl side of basic amino acids, are able to abolish completely and irreversibly the proteolytic and esterolytic activities of trypsin. Leupeptin and antipain have been reported as efficient inhibitors of trypsins from menhaden (Pyeun et al., 1990), catfish trypsin (Yoshinaka et al., 1984) and capelin (Hjelmeland and Raa, 1982).

Trypsins from krill (Osnes and Mohr, 1985b), cod (Simpson et al., 1989) and menhaden (Pyeun et al., 1990) were almost entirely inhibited by benzamidine, but trypsin from shrimp (Honjo et al., 1990) was not inactivated even though higher concentration of benzamidine was applied. Thus, inhibition of trypsin by benzamidine may vary with the source of enzyme.

Soybean trypsin inhibitor (SBTI) and diisopropylfluorophosphate (DFP) have been described as strong inhibitors of trypsin and chymotrypsin from crustacea and fish intestine including, Atlantic blue crab (Dendinger and O'Connor, 1990), shrimp (Honjo et al., 1990), Antarctic krill (Osnes and Mohr, 1985b), catfish (Yoshinaka et al., 1984), and menhaden (Pyeun et al., 1990). Phenylmethanesulfonyl fluoride (PMSF) sulfonylates protein exclusively at active site histidine in the trypsin molecule. The chemical had various inhibitory effects for menhaden trypsin (Pyeun et al., 1990), catfish

trypsin (Yoshinaka et al., 1984), cod trypsin (Simpson and Haard, 1984a; Simpson et al., 1989), anchovy trypsins (Martinez et al., 1988), and capelin trypsins (Hjelmeland and Raa, 1982).

Inhibition of fish trypsin by ethylenediamine tetraacetate (EDTA), a metal chelating agent, varies with source of trypsin. Crustacean trypsin was inhibited approximately 20 to 35% (Galgani et al., 1984; Galgani and Nagayama, 1987a,b). Two trypsins from menhaden were nearly completely inactivated (Pyeun et al., 1990) and two anchovy trypsins evidenced 34 and 60% inactivation (Martinez et al., 1988). However, Winter and Neurath (1970) reported that EDTA slightly in 15% activated a trypsin from starfish.

Disulfide bonds are stable and important to stabilizing protein conformation by increasing protein hydrophobicity. Addition of reducing agents will break disulfide bonds and cause inactivation of enzymes. Wolz and co-worker (1990) reported that sulfhydryl reagents had a strong inhibitory effect on Astacus protease in the order of dithiothreitol, 2-mercaptoethanol and glutathione. Also, two trypsins from anchovy intestine were inhibited with 2.5 mM mercaptoethanol (Martinez et al., 1988). However, trypsins from five species of shrimp were not affected by 2 mM mercaptoethanol (Galgani and Nagayama, 1987a).

5. Utilization of Seafood Byproduct

Studies have demonstrated crustacean byproduct utilization including astaxanthin pigments from shrimp waste (Torrissen et al., 1981) and crawfish waste (Chen and Meyers, 1982a, b; Meyers and Chen, 1985). Extraction of astaxanthin from crawfish waste has resulted in a commercial process. Feed produced partially with dried meal from crawfish and shrimp increased natural pigmentation in salmonoids and egg (Lee, 1986). Chitosan from crawfish shell has commercial feasibility as a coagulant in the recovery of organic compounds from seafood processing water (No and Meyers, 1989).

Another study has indicated the potential for recovery of water soluble organics in discharge streams from shrimp processing plants (Perkins and Meyers, 1977). Proteinaceous solids recovered from shrimp peeler effluent by HCl precipitation and centrifugation reduced supernatant total organic nitrogen and biochemical oxygen demand (Depaola et al., 1989). The process for the recovery of organic materials from waste water not only solves pollution problems but also maximally utilizes wastes for ultimate human consumption.

Wash water from clam processing plants has been converted into a potentially marketable clam juice (Hood et al., 1976), a dehydrated clam flavor ingredient (Joh and Hood, 1979), a protein concentrate (Hang et al., 1980), and a natural clam flavoring agent (Burnette et al., 1983). Reddy et al. (1989) studied clam processing wash waters for utilization as

flavoring agents and evaluated their potential as marketable by-products or food ingredients. Shiau and Chai (1990) concentrated oyster shucking liquid wastes, which contains valuable protein, nonproteinaceous nitrogen compounds and other organic materials, and evaluated a potential source of by-products for human consumption as oyster soup.

Tanchotikul and Hsieh (1989) analyzed and compared flavor components obtained from crayfish waste with those identified in crayfish tail meat and hepatopancreas. Also, Baek et al. (1991) have prepared flavor concentrates from crayfish processing waste at different temperatures and found that higher levels of pyrazine compounds were produced at higher thermal concentration temperatures. Cha et al. (1991) have reported that crayfish concentrate has potential for industrial uses as a crawfish flavor base.

Crab waste, which contains 31-32% protein (dry wt basis) and is made up of approximately 40% essential amino acids, has potential for utilization in various animal feeds, as microbial growth media and other food products after acid hydrolysis (Jaswal, 1990). Blue crab waste that remains after processing is currently under investigation in the Food Science Department, Louisiana State University, as a source of value-added seafood products.

Konigsbacher and Hewitt (1964) reported that flavor development in living organisms is related to enzyme-catalyzed metabolic pathways. The production of savory flavors by

enzymes in vitro is not well characterized and are more often associated with the production of bitter taste by hydrophobic amino acids. The use of proteases with different bond specificities has not been thoroughly investigated but has potential in flavor production. Proteolytic enzymes from crayfish hepatopancreas (Kim et al., 1989a) and menhaden (Pyeun et al., 1989) have been studied for potential industrial applications. Incorporation of enzymes from fish or crustacean viscera may improve the extraction and modification of volatile flavor compounds from crab waste.

CHAPTER III

MATERIALS AND METHODS

1. Purification of Anionic Trypsins from Crawfish Hepatopancreas

A. Analytical Approach

The objective of this experiment was to evaluate the existence of major proteolytic enzymes in the crawfish hepatopancreas. The major proteases identified in the crude extract was found to have high tryptic activity. Therefore, a purification scheme for trypsin-like enzymes from crawfish hepatopancreas was established to evaluate physicochemical properties that could be responsible for the degradation of the tail meat.

B. Sample Collection and Preparation

Live crawfish (Procambarus clarkii) was generously donated by Klein's Seafood Inc., Port Allen, LA., and transported to the LSU Department of Food Science. The hepatopancreas from live crawfish was collected, frozen immediately in a liquid nitrogen cabinet freezer (Model F831059E, Air Products & Chemicals Inc., Allentown, PA), and stored at -85°C until used for crude enzyme extraction.

C. Extraction of Enzyme from Crawfish Hepatopancreas

Four hundred ml of 1% sodium chloride solution containing 1 mM Na_2EDTA was added to 200 g of the crawfish hepatopancreas and homogenized with a Tissumizer (Tekmar Co. Cincinnati, OH) for 30 sec. The extract was centrifuged at $12,000 \times g$ for 20 min at 4°C and the supernatant was carefully filtered through Whatman No. 1 filter paper. The filtrate was dialyzed against distilled water overnight and centrifuged at $12,000 \times g$ for 20 min. The crude enzyme solution was obtained by collecting the supernatant after centrifugation.

D. Determination of Enzyme Activity

1) Preparation of Buffer Solutions

Buffer solutions for determination of caseinolytic activity were prepared with four sequences as follows; 0.2 M glycine-0.1 M HCl (pH 1.8-3.2), 0.1 M citrate-0.2 M Na_2HPO_4 (pH 3.0-7.0), 0.1 M Tris-HCl (pH 7.0-9.0), and 0.1 M Na_2CO_3 - NaHCO_3 (9.0-11.0).

The concentration of all buffers was 0.05 M, which were then used for determination of the effect of pH on esterase and amidase activity. Phosphate buffer was substituted for N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) to prevent the formation of calcium phosphate. Activities were determined in different buffers with overlapping pH points to exclude the possibility of an influence exerted by the nature

of the buffer species.

2) Preparation of Casein and Hemoglobin Solution

Casein Solution: Five g of casein (C5890, Sigma, MO) was dissolved in 40 ml of 0.2 M Tris-HCl buffer, pH 8.0, for 3 hrs and then brought to a volume of 100 ml with distilled water. The suspension was heated for 5 min in boiling water to denature casein. This solution was stored at refrigerated temperature (4°C) and prepared weekly to minimize possible microbial contamination.

Hemoglobin Solution: Five g of hemoglobin (H2625, Sigma, MO) was placed in a 100 ml volumetric flask and brought to volume with 0.06 N HCl. The suspension was dialyzed against distilled water overnight.

3) Determination of Protein Concentration

Protein concentration was determined by the method described in the Bio-rad manual (1988) using bovine serum albumin as a standard. Protein concentrations of crude enzyme and ammonium sulfate fraction were determined after filtration through a 0.2 μ m disposable syringe filter (Anotop, UK).

4) Determination of Protease Activity

Caseinolytic activity of the crude enzyme solution was measured by a modification of the method of Anson (1938). The assay mixture was composed of 0.75 ml of buffer solution, 0.25

ml of 5% casein solution, and 50 μ l of enzyme solution. The reaction mixture was incubated for 20 min and reaction was stopped by addition of 1.25 ml of 5% (W/V) trichloroacetic acid (TCA) solution. After standing for 30 min at ambient temperature, the solution was centrifuged at 3,000 \times g for 15 min and the supernatant assayed for TCA-soluble peptides according to the modified method of Lowry et al. (1951). To 0.5 ml of supernatant solution was added 2.0 ml of 0.55 M sodium carbonate solution and 0.5 ml of 1.0 N Folin & Ciocalteu's phenol reagent (F9252, Sigma, MO). The mixture was incubated at 30°C for 20 min and absorbance at 660 nm determined. A blank was obtained by adding TCA solution to the substrate prior to addition of enzyme. The absorbance was converted to mmole of tyrosine using a calibration curve (Appendix 1). Caseinolytic activity (U/min/ml) was defined as the absorbance equivalent of 1 mmole tyrosine produced per min per ml of enzyme solution under experimental conditions. Specific activity (U/min/mg) was expressed as the absorbance equivalent of 1 mmole tyrosine produced by 1 mg of protein per 1 min by compensating for protein concentration of sample solution. In this study, one unit of caseinolytic activity was calculated with the following formula:

$$1 \text{ Casein Unit} = \frac{A_{660} \times 4.6 \times 0.615}{0.05 \text{ ml} \times 20 \text{ min} \times \text{protein concentration}}$$

where 0.615 is the conversion factor of the absorbance

equivalent to 1 mmole tyrosine obtained from Appendix 1 and 4.6 is a dilution factor.

5) Determination of Collagenase Activity

Collagenolytic activity was determined by a modification of the method of Mandl et al. (1953). Twelve mg of bovine achilles tendon collagen (Type I, C9879, Sigma, MO) was suspended in 2.5 ml of 0.1 M TES [tris(hydroxymethyl)-methyl-2-aminoethane sulfonate] buffer (pH 7.5) containing 0.35 mM CaCl_2 at 37°C for 20 min. Reaction was started by addition of 50 μl of enzyme solution to the suspended collagen. After 5 hr incubation, the reaction mixture was centrifuged at 4,000 $\times g$ for 5 min and 0.2 ml of supernatant was transferred to test tubes and 0.5 ml of cyanide-acetate buffer, and 0.5 ml of 3% ninhydrin solution was added. This solution was heated for 15 min at 100°C and after cooling, was diluted with 2.5 ml of 50% isopropyl alcohol and shaken vigorously. The mixture was cooled to room temperature and absorbance measured at 600 nm. The absorbance was converted to mmoles of leucine using a calibration curve (Appendix 2). A blank was obtained by mixing collagen in buffer solution and incubating for 5 hr. Enzyme solution was incubated for 5 hr in a separate test tube. Just prior to addition of ninhydrin solution, enzyme and collagen solution were combined and treated as described for test solutions. Collagenolytic activity (U/hr/ml) was defined as the absorbance equivalent of 1 mmole leucine produced per hr

per ml of enzyme solution at pH 7.5 and 37°C. Specific activity (U/hr/mg) was expressed as the absorbance equivalent of 1 mmole leucine produced by 1 mg of protein per 1 hr with compensation for protein concentration of sample solution. In this study, one collagenolytic activity unit was calculated with the following formula:

$$1 \text{ Collagen Unit} = \frac{A_{600} \times 12.75 \times 0.556}{0.05 \text{ ml} \times 5 \text{ hr} \times \text{protein concentration}}$$

where 0.556 is the conversion factor of the absorbance equivalent to 1 mM leucine obtained from Appendix 2 and 12.75 is a dilution factor.

6) Determination of Esterase Activity with TAME and BAEE

Experiments were carried out under steady state conditions with an excess of substrate. The rate of hydrolysis of N-p-tosyl-L-arginine methyl ester (TAME, T4626, Sigma, MO) by enzyme was monitored by measuring the liberation of N-p-tosyl-L-arginine at 247 nm. The reaction was performed in a thermocuvette using a Gilford response UV-VIS Spectrophotometer (Gilford Ins. Lab. Inc., OH) with temperature controlled by an Endocol RTE-9 refrigerated circulator (Neslab Ins. Inc., NH). Monitoring of tryptic activity during purification was performed with 1 mM TAME at pH 8.1 and 25°C by modification of the method described by

Worthington Biochemical Co. (Anon, 1988). Twenty-five μ l of an appropriately diluted enzyme solution was mixed with 0.5 ml of 1 mM TAME, equilibrated to 25°C, dissolved in 0.05 M Tris-HCl buffer, pH 8.1, containing 1 mM CaCl_2 . The activity was measured at 247 nm for 3 min. One TAME unit (U/min/ml) was defined as the amount of enzyme that hydrolyzed 1 mmole of TAME per min per ml of enzyme solution under the conditions described above. Specific activity (U/min/mg) was expressed as enzyme units per mg of protein. In this study, one TAME activity unit was calculated with the following formula:

$$1 \text{ TAME Unit} = \frac{A_{247} \times 1000 \times 0.525}{540 \times 0.025 \times \text{protein concentration}}$$

where 540 is the extinction coefficient of 1 M N-p-tosyl-L-arginine relative to N-p-tosyl-L-arginine methyl ester at 247 nm.

The rate of hydrolysis of benzoyl L-arginine ethyl ester (BAEE, B4500, Sigma, MO) was monitored by measuring liberation of benzoyl L-arginine with absorbance at 253 nm. Reaction was performed using the condition described previously. One BAEE unit (U/min/ml) was defined as the amount of enzyme that hydrolyzed 1 mmole of BAEE per min per ml of enzyme solution. Specific activity (U/min/mg) was expressed as enzyme units per mg of protein. In this study, one BAEE activity unit was calculated with the following formula:

$$1 \text{ BAEE Unit} = \frac{A_{233} \times 1000 \times 0.525}{1048 \times 0.025 \times \text{protein concentration}}$$

where 1048 is the extinction coefficient of 1 M benzoyl-L-arginine relative to benzoyl arginine ethyl ester at 247 nm.

Chymotrypsin activity also was determined by change in absorbance at 256 nm for hydrolysis of benzoyl-L-tyrosine ethyl ester (BTEE, B6125, Sigma, MO), or at 238 nm for hydrolysis of acetyl-L-tyrosine ethyl ester (ATEE, A6751, Sigma, MO) (Hummel, 1959). The extent of hydrolysis was calculated from the respective change of the absorbance due to hydrolysis as follows;

$$1 \text{ BTEE Unit} = \frac{A_{256} \times 1000 \times 0.525}{964 \times 0.025 \times \text{protein concentration}}$$

where 964 is the extinction coefficient of 1 M benzoyl-L-tyrosine relative to benzoyl-L-tyrosine ethyl ester at 256 nm.

$$1 \text{ ATEE Unit} = \frac{A_{238} \times 1000 \times 0.525}{2250 \times 0.025 \times \text{protein concentration}}$$

where 2250 is the extinction coefficient of 1 M acetyl-L-tyrosine relative to acetyl-L-tyrosine ethyl ester at 238 nm.

7) Determination of Amidase Activity with BAPNA

Amidase activity was measured as hydrolysis of benzoyl-D,L-arginine p-nitroanilide (BAPNA, B4875, Sigma, MO) with the

assay method of Erlanger et al. (1961). Twenty-five μ l of enzyme solution was mixed with 0.5 ml of 1 mM BAPNA dissolved in 0.05 M Tris-HCl buffer, pH 8.1, containing 1 mM CaCl_2 . The hydrolysis of BAPNA was measured at 410 nm at 45°C during purification because of low amidase activity in ion and gel chromatographic fractions. One enzyme unit was defined as the amount of enzyme that hydrolyzed 1 mmole of BAPNA per min under the conditions described above. Specific activity was expressed as enzyme units per mg of enzyme.

$$1 \text{ BAPNA Unit} = \frac{A_{410} \times 1000 \times 0.525}{8,800 \times 0.025 \times \text{protein concentration}}$$

where 8,800 is the extinction coefficient of 1 M p-nitroanilide relative to benzoyl-D,L-arginine p-nitroanilide.

E. Isolation of Anionic Trypsins from Crawfish Hepatopancreas

The measurement of trypsin activity throughout enzyme purification was monitored using TAME as a substrate and secondarily with BAPNA and casein as substrates. Esterase and amidase activity were done using 1.0 mM TAME and BAPNA, respectively, at pH 8.1 and 25°C for TAME, or 45°C for BAPNA. The crude enzyme and acetone fractions for esterase and amidase activity and for the protein assay were filtered through 0.5 μ m disposable syringe filters (Anotop, UK). Caseinolytic activity was measured at 45°C and pH 6.8 using 5% casein solution as substrate. As presented in Appendix 3,

purification of proteases from crude enzyme solution was undertaken by first employing acetone fractionation with 30-60% saturation. The acetone fraction was dialyzed against 0.02 M Tris-HCl buffer, pH 6.8, containing 0.5 M NaCl, 5 mM CaCl₂, and 1 mM benzamidine to prevent autolysis of the proteases during the chromatographic process. After dialysis overnight against the same buffer, the sample was applied to a benzamidine-Sepharose 6B column (1.5 x 10 cm) equilibrated with the above buffer without benzamidine. The column was eluted with the equilibration buffer until the effluent did not contain protein. Trypsin-like enzymes were then eluted from the column using 0.02 M Tris-HCl, pH 6.8, containing 0.5 M NaCl, 5 mM CaCl₂, and 125 mM benzamidine. The trypsin fractions were pooled and dialyzed against 0.02 M Tris-HCl buffer, pH 6.8, and applied to a DEAE-Sephadex column (3 x 40 cm) equilibrated with the same buffer solution. The column was eluted with a 2,000 ml linear gradient ranging from 0 to 2.0 M sodium chloride. Trypsin A and B fractions were rechromatographed with a second DEAE-Sephadex column (2.0 x 20 cm) equilibrated with the same buffer solution. The column was eluted with a 1,000 ml linear gradient ranging from 0.5 to 1.5 M sodium chloride. Fraction A and B were separated into two different protease fractions with the second DEAE-Sephadex chromatography. Each tryptic fraction was concentrated with ultrafiltration using a 10,000 molecular weight cut off (Sartorius, W. Germany) filter and dialyzed against 0.02 M

Tris-HCl, pH 6.8, containing 0.1 M NaCl. The dialyzed solution was applied to a Sephacryl S-200 column (2.5 x 90 cm) equilibrated with the same buffer, pH 6.8. Fractions with high tryptic activity were concentrated with ultrafiltration and stored at -20°C until used in subsequent characterization studies.

F. Electrophoresis

Discontinuous polyacrylamide gel electrophoresis (Disc-PAGE) at pH 8.3 using 20 mM Tris-glycine buffer was carried out according to the method of Davis (1964). Sample was loaded onto a 7.5% or 10% polyacrylamide gel column (0.6 x 10 cm) with electrical current of 1 mA per column. Stacking gel (3%) was prepared with an acrylamide to bis (acrylamide) ratio of 4:1 and buffered with 62.5 mM Tris-HCl, pH 6.8. The samples were dialyzed against 62.5 mM Tris-HCl buffer, pH 6.8, containing 15% glycerol at 4°C before electrophoresis. After electrophoresis, the gel was fixed with 25% isopropanol/10% acetic acid for 3 hr then stained for 5 hr with 0.01% Coomassie Brilliant Blue R-250 in 50% methanol/10% acetic acid. The gel was destained in methanol: acetic acid:water (3:1:6) until the background was clear.

SDS-polyacrylamide gel electrophoresis was done according to the method of Laemmli (1970) using 10% gel with a constant current of 1 mA per sample. Electrophoresis was carried out at pH 8.3 with 25 mM Tris-192 mM glycine buffer containing 0.1%

SDS. The procedure of staining and destaining were the same as described previously.

G. Elution of Enzyme from Electrophoretic Gel

Eight tubes of gels to which purified trypsins were applied were subjected to disc-PAGE at pH 8.3 as described previously. Immediately after termination of electrophoresis, the gels were removed from the tubes and one gel of each trypsin was stained as described previously. The other four unstained gels were cut into 3 mm slices to separate enzymes. Each slice was transferred to a tube containing 0.5 ml of 0.1 M Tris-HCl buffer, pH 8.1, containing 1.0 mM calcium chloride. The gel slices were homogenized with a glass rod to extract trypsin from acrylamide gel and centrifuged for 10 min at 4°C. The supernatant was used for determination of esterolytic activity with 1 mM TAME to compare protein bands in stained gel.

H. Molar Extinction Coefficient of Trypsins

Each enzyme solution was diluted with distilled water to 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg of protein per ml. Diluted protein solution was measured for absorbance at 280 nm and the absorbance was converted to molar extinction coefficient by compensation for each molecular weight.

I. Estimation of Molecular Weight by Gel Filtration

Molecular weight of the purified proteinase was determined by Sephacryl S-200 gel filtration according to the method of Andrews (1964) using bovine albumin (M.W. 66,000), carbonic anhydrase (M.W. 29,000), cytochrome C (M.W. 12,400), and aprotinin (6,500) as standard proteins.

J. Isoelectrofocusing

Isoelectrofocusing of purified proteases was performed using a mini-isoelectric focusing cell (Model 111 mini IEF cell, Bio-rad, CA) with a pH 4.65-9.0 of agarose isoelectric focusing gel (Isogel, FMC). After isoelectrofocusing, the gel plate was soaked for 10 min in fixing solution (36% methanol, 0.367 M trichloroacetic acid and 0.142 M of sulfosalicylic acid) and then rinsed with distilled water, blotted with Whatmann 3 MM blotting paper, and dried with hot air. The dried gel was stained for 20 min with Coomassie Brilliant Blue R-250 solution (0.1% CBB, 25% ethanol and 9% acetic acid) and destained for 3 min in destaining solution.

K. Determination of Amino Acid Composition

The purified enzymes were hydrolyzed in 6 N HCl for 24, 48 and 72 hr at 110°C in sealed tubes evacuated to 0.01 mmHg pressure in a Pico-Tag Work Station (Waters, CA, USA). The Amino acid composition was determined using a Waters HPLC Pico-Tag system with a reverse-phase C₁₈ column. The residues

of serine and threonine were calculated with extrapolation of the data obtained from 24, 48, and 72 hr hydrolysis.

2. Enzymatic Characterization of Crawfish Hepatopancreatic Proteases

A. Analytical Approach

These experiments were performed to characterize enzymatic properties of purified trypsins from crawfish hepatopancreas. Also, stability of trypsins for industrial utilization and their activity toward structural proteins in tail meat were evaluated.

B. Effect of pH on Trypsin Activity

The effect of pH on hydrolysis of casein was measured with purified proteases (enzyme concentration; 100 $\mu\text{g/ml}$) using 5% casein at pH values in the range of 4.0 to 10.5. The rates of casein hydrolysis by purified trypsins were constant up to 20 min incubation (Appendix 4), thus caseinolytic activities with purified trypsins were determined by 20 min incubation. The esterolytic and amidolytic activity using 1 mM TAME or 1 mM BAEE (enzyme concentration, 10 $\mu\text{g/ml}$) and 1 mM BAPNA (enzyme concentration; 100 $\mu\text{g/ml}$) as substrates, respectively, were determined at pH values ranging from 4.0 to 10.0. Activities were determined in different buffers with overlapping pH points to exclude the possibility of an influence exerted by the nature of the buffer solution.

C. Effect of Temperature on Trypsin Activity

The optimum temperature was measured at pH 6.8 and 8.1 with 1 mM TAME for esterolytic activity and 1 mM BAPNA for amidolytic activity. A pH of 6.8 was used with 5% casein for proteolytic activity. A temperature range of 25 to 70°C was employed with the same concentration of enzyme solution described previously.

D. Determination of pH Stability

Purified enzyme solutions (20 µg/ml) were incubated at 25°C for 8 hr in the same volume of various buffers containing 1 mM CaCl₂. Buffers included 0.1 M sodium citrate-citrate (pH 4.0 and 5.6), 0.1 M TES-Na-TES (pH 6.8), 0.1 M Tris-HCl (pH 8.1) and 0.1 M Na₂CO₃-NaHCO₃ (pH 10.5). At different times, aliquots were withdrawn from the incubated samples and assayed for esterolytic activity at pH 8.1 using 1 mM TAME as substrate.

E. Determination of Thermal Stability

Thermal stability was measured through incubation of the enzyme in 50 mM Tris-HCl buffer, pH 8.1, containing 1 mM CaCl₂, at various temperatures for 5 min. The remaining esterolytic activities were determined using 1 mM TAME as substrate at pH 8.1 and 25°C.

The stabilizing effect of protein on tryptic activity was studied by adding 5% casein solution to the purified trypsin

solutions before preheating. Treatment involved heating in 0.25 ml of 0.1 M Tris-HCl (pH 6.8) and in 0.25 ml of 5% casein solution (0.1 M Tris-HCl, pH 6.8). Following this, remaining proteolytic activity was measured at 45°C and pH 6.8 for 20 min. To compensate for casein hydrolysis during heat treatment in the test containing casein solution, a reference test was performed by adding TCA solution to tubes containing casein solution after heat treatment and the amount of casein hydrolysis during heat treatment was then subtracted from the value of the test solution.

F. Effect of Calcium Ion on Trypsin Activity

Experiments requiring metal-free conditions were carried out with glassware soaked in 30% nitric acid and then rinsed carefully with deionized water. Metal-free dialysis tubing was prepared by extensive washing with metal-free water at 80°C (Auld, 1988). Disposable metal-free polyethylene pipet tips were used for all assays. Apo-proteases were prepared by the procedure developed for carboxypeptidase A (Wagner, 1988). A 0.5 ml solution of enzyme (50 µg/ml) in 50 mM Tris-HCl buffer (pH 8.1) was dialyzed for 4 days at 4°C against four changes of 100 ml of the same buffer containing 1 mM ethylenediamine-tetraacetate disodium (2Na-EDTA). Subsequently, excess chelator was removed by dialysis versus three changes of 200 ml of metal-free buffer over 3 days. The apoenzyme was stored in solution at 4°C and assayed for esterolytic activity using

1 mM TAME as substrate in the presence of various concentration of CaCl_2 .

G. Effect of Chemical Reagents on Trypsin Activity

Phenylmethylsulfonyl fluoride (PMSF) inhibition of trypsin was studied using a modification of the procedure of Fahrney and Gold (1963). PMSF was dissolved in 5% 2-propanol to a final concentration of 1 mM, and equal volumes of the purified enzyme solutions were incubated separately with equal volumes of PMSF solution at 25°C for 30 min. After incubation, residual esterolytic activity was assayed with 1 mM TAME at pH 8.1.

Soybean trypsin inhibitor (SBTI) was dissolved in deionized water to concentrations of 1.0 and 5.0 μM . The trypsin solutions were added separately to equal volumes of SBTI solutions and incubated at 25°C for 15 min. After incubation, residual esterolytic activity was determined with 1 mM TAME.

Other inhibitors, such as N_α -p-tosyl-L-lysine chloromethyl ketone (TLCK), antipain, benzamidine, diisopropyl fluorophosphate (DFP), ethylenediamine tetraacetate disodium (2Na-EDTA), iodoacetate, p-chloromercuribenzoate and sulfhydryl reagents, i.e., dithiothreitol (DTT), glutathione, and mercaptoethanol were dissolved in D.W. and used in this study. Also N_α -p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), leupeptin, pepstatin and o-phenanthroline were

dissolved in dimethyl sulfoxide and diluted with D.W. to 1 mM concentration. Tryptic activity was determined with 1 mM TAME dissolved in 50 mM Tris-HCl containing 1 mM CaCl₂ (pH 8.1) after 15 min incubation at 25°C with the same volume of each trypsin solution (20 µg/ml).

H. Isolation of Myofibrillar Proteins from Crawfish Tail Meat

Ten g of chopped crawfish tail meat was homogenized with 10 volumes of KCl-phosphate buffer ($\mu=0.05$, pH 7.2) with a Tissumizer (Tekmar Co. Cincinnati, USA) for 1 min. Homogenate was centrifuged at 5,000 x g for 20 min and the precipitate was further separated for myofibrillar proteins. Precipitate was dissolved in 10 vol. of KCl-phosphate buffer ($\mu=1.0$, pH 7.2) and suspended for 3 hr at 4°C. The solution was centrifuged at 5,000 x g for 20 min and supernatant was resuspended in 9 vol of distilled water. The suspension was adjusted to pH 6.6 with 10% acetic acid and centrifuged at 10,000 x g for 15 min. The precipitate was dissolved with 3 M KCl to a final ionic strength of 0.5, and 9 vol. of D.W. was added to give a final ionic strength of 0.05. After stirring for 3 hr in a cold room (4°C), the suspension was centrifuged at 10,000 x g for 15 min and the precipitate removed. This step was repeated 3 times. The final precipitate was dissolved in KCl and dialyzed against 0.5 M KCl-phosphate containing Tris-maleate (20 mM in total solution) overnight. Crawfish

myofibrillar proteins were obtained from the supernatant solution after centrifugation at 12,000 x g for 15 min. Myofibrillar protein solution was denatured by dialyzing against 8 M urea overnight and dialyzed against D.W. for 3 days before using as substrate for proteolytic activity.

I. Isolation of Sarcoplasmic Proteins from Crawfish Tail Meat

Sarcoplasmic protein was prepared using a modification of the procedure of Yates and Greaser (1983). Ten g of chopped crawfish tail meat was homogenized in 10 vol of 15 mM sodium phosphate buffer (pH 7.2) containing 70 mM KCl and 5 mM β -mercaptoethanol using a tissumizer for 30 sec. The homogenate was centrifuged at 800 x g for 15 min. The supernatant was removed and centrifuged at 25,000 x g for 30 min. The clarified supernatant was adjusted to pH 1.0 to inactivate proteolytic activity and then dialyzed against distilled water overnight. The dialysate was used as the substrate for subsequent proteolytic activity by purified proteases.

J. Proteolysis of Myofibrillar Proteins

To determine the hydrolysis of myofibrillar proteins or sarcoplasmic proteins by crawfish trypsins, the assay mixture was composed of 0.5 ml of buffer, 0.5 ml of protein solution (10 mg/ml for myofibrils, 5 mg/ml for sarcoplasmic proteins) and 50 μ l of enzyme solution (100 μ g/ml). The reaction conditions and assay procedures were the same as described in

the determination of caseinolytic activity.

Myofibrillar protein degradation also was evaluated with electrophoresis using a modified method of Busconi et al. (1989). Myofibrils (3.0 mg) were suspended in 0.1 M Tris-HCl buffer, pH 7.5, and incubated with 25 μ l of enzyme solution (100 μ g/ml) at 25°C. After different incubation times, the reaction was stopped by the addition of 1.0 ml of electrophoresis sample buffer (8 M urea, 2.5% SDS, 5 mM EDTA, 1% 2-mercaptoethanol in 0.1 M Tris-glycine buffer, pH 8.8) and boiled for 2 min. Aliquots containing 50 μ g of original myofibrillar protein were analyzed by SDS-PAGE as described previously.

3. Kinetic and Thermodynamic Properties of Trypsins

A. Analytical Approach

The purpose of these experiments was to elucidate and compare the kinetic and thermodynamic properties of crawfish trypsins in esterolytic, amidolytic, and caseinolytic reactions.

B. Determination of Kinetic Parameters

Apparent Michaelis-Menten constant (K_m') and substrate turnover number (V_{max}) were determined by least-squares regression analysis. Kinetic data with TAME as a substrate for esterase reaction were obtained with substrate concentrations

of 0.2, 0.25, 0.4, 0.5, and 1.0 mM TAME at pH 6.8 and 8.1 at a temperature of 25°C. Substrate concentration of BAEE for esterase reaction and reaction condition were 0.05, 0.067, 0.1, and 0.2 mM BAEE, pH 8.1 and 25°C. Kinetic data with BAPNA as a substrate for amidase reaction at pH 6.8 were obtained with the same substrate concentration as TAME, however, at pH 8.1 substrate concentrations of 0.025, 0.033, 0.05, and 0.01 mM of BAPNA and 45°C were used. Kinetic data with casein as a substrate for protease reaction were obtained with substrate concentrations of 0.2, 0.25, 0.5, and 1.0% casein for both pHs and temperature of 45°C. The physiological efficiency for trypsins was defined as the ratio V_{max}/K_m' . Molar concentrations of the enzymes were estimated using molecular weights determined by gel filtration and protein concentration.

C. Determination of Thermodynamic Parameters

The experimental activation energy (E_a) was calculated from the slope of the line obtained by Arrhenius plot using log specific activity against $1/T$ (slope = $-E_a/2.303R$). The fit of the data to the Arrhenius equation was evaluated by least-squares regression analysis.

4. Application of Crawfish Enzymes as an Extractant in Recovery of Protein and Flavor Compounds from Seafood Waste

A. Analytical Approach

The objective of these experiments was to evaluate the potential of crab waste as a source of crab flavor base using crawfish enzyme as an extraction aid. Volatile flavor compounds from crab waste were analyzed and a comparison was made between enzyme treated and untreated crab waste.

B. Collection of Crab Waste

Picked crab waste was transported from a local seafood processor to the laboratory under ice. After 1 day at refrigeration temperature (4°C), the waste was minced with a muscle/shell separator (Model 23-688, Stephan Paoli International, Rockford, IL) at the LSU Muscle Food Processing Lab. Protein and flavor compounds were extracted from the shell particle generated through mincing. The sample was divided into approximately 500 g portions and packaged in polyethylene bags for frozen storage at -20°C until further studies.

C. Chemical Composition of Crab Waste

Values for nitrogen, ash, fat and moisture content were averaged in triplicate on a wet basis. Nitrogen was determined by a semiautomated method (AOAC, 1980), and ash, fat, and

moisture by Standard Methods (AOAC, 1980).

D. Optimum Condition for Hydrolysis of Crab Waste Using Crawfish Crude Extract

1) Effect of Enzyme Concentration on Hydrolysis of Crab Waste Protein

Hydrolysis of crab waste was accomplished using a modification of the method described previously in the caseinolytic activity section. The reaction mixture was composed of 10 g of crab waste, 15 ml of buffer solution and 0.5 to 2 ml of crawfish crude extract. The reaction mixture was incubated for 4 hr at 45°C and pH 8.0. The reaction mixture was then centrifuged for 15 min at 4°C and 8000 x g. After centrifugation, aliquots were taken from the supernatant solution for protein assay and amino group analysis. The remaining supernatant solution was boiled for 1 hr to inactivate enzymes and to develop heat-induced flavor and taste compounds. Protein content was determined by the method described in the Bio-rad manual (1988) using bovine serum albumin as a standard. The amount of total amino group containing compounds was determined according to the modified method of Lowry et al. (1951). To 0.5 ml of supernatant was added 2.0 ml of 0.55 M sodium carbonate solution and 0.5 ml of 1.0 N Folin & Ciocalteu's phenol reagent (F9252, Sigma, MO). The mixture was incubated at 30°C for 20 min, after which absorbance at 660 nm was determined and converted to mmole of

tyrosine using a calibration curve (Appendix 1).

2) Effect of Reaction Time on Hydrolysis of Crab Waste Protein

The optimum reaction time for hydrolysis of crab waste protein was measured using the range of 0.5 to 4 hr incubation at 45°C and pH 8.0. After the appropriate time interval, reaction mixture was centrifuged at 8,000 x g for 15 min and aliquots were taken from the supernatant solution for protein and amino group analysis. Optimum reaction time was determined by comparing the amount of amino groups and protein liberated.

3) Effect of pH on Hydrolysis of Crab Waste Protein

The optimum pH for waste protein hydrolysis was measured at 45°C over the pH range of 6.0-10.5. Buffers used were 0.05 M Na_2HPO_4 - Na_7HPO_4 for pH 6.0-8.0, 0.05 M Tris-HCl for pH 8.0-9.0, and 0.05 M Na_2CO_3 - NaHCO_3 for pH 9.0-10.5.

E. Flavor Analysis of Recovered Crab Waste

1) Extraction of Flavor compounds from Crab Waste

Two hundred and fifty g of crab waste was mixed with 10 ml of the acetone fraction of crude enzyme from crawfish hepatopancreas with forced circulation by impeller in 500 ml of 10 mM Na_2PO_4 - NaHPO_4 (pH 9.0) for 4 hr at 45°C. A reference was obtained using 10 ml of distilled water instead of enzyme.

After incubation, the reaction mixture was filtered through one fold of cheese cloth using a Buchner funnel. The filtrate was immediately heated at 100°C for 1 hr for heat-induced flavor development and enzyme inactivation.

2) Simultaneous Steam Distillation-Solvent Extraction (SDE)

One and a quarter liter of distilled water, 750 ml of crab flavor extract and 2.0 ml of an aqueous solution of internal standard 2,4,6-trimethylpyridine (45.392 μ g) were added to a 5-L round-bottom flask, which was connected to the heavier-density-solvent arm of a Likens-Nickerson (Likens and Nickerson, 1964) simultaneous steam distillation/solvent extraction (SDE) apparatus (Kontes, Vineland, NJ). One-hundred ml of redistilled diethyl ether was used as an extracting solvent in a 100 ml round-bottom flask attached to the lighter-density-solvent arm. After extraction for 4 hrs, the solvent was collected and kept at -20°C overnight to remove ice crystals and transferred to a 100 ml volumetric flask. The solvent was evaporated under a gentle stream of nitrogen to approximately 10 ml and dried over 2 g of anhydrous sodium sulfate. Final volume was reduced to 0.2 ml with a gentle nitrogen gas stream in a conical tube. Extractions were carried out in duplicate for each treatment.

3) Gas Chromatography/Mass Spectrometry (GC/MS)

Five μ l of each SDE extract was analyzed with an HP 5792A GC/5970B mass selective detector (MSD) (Hewlett-Packard Co., Palo Alto, CA). Volatile components were separated with a fused silica capillary column (Supelcowax 10; 60 m length x 0.25 mm i.d. x 0.25 μ m film thickness; Supelco, Inc., Bellefonte, PA). The linear velocity of helium carrier was 25.7 cm/s. Oven temperature was programmed from 40°C to 175°C at a rate of 2°C/min with initial and final hold times at 5 and 30 min, respectively. Oven temperature was further increased to 195°C at a rate of 5°C/min and maintained for 25 min. Electron ionization mass spectra were obtained under the following conditions; mass range of m/z at 33-300, ionization energy at 70 eV, electron multiplier voltage at 2000 V, and scan rate of 1.60 scans/sec. Other details of the MSD procedure have been previously described by Vejaphan et al. (1988). Duplicate analyses were performed on each SDE extract.

4) Compound Identifications

Volatile compounds were identified by matching retention indices (RI), calculated according to van den Dool and Kratz (1963), and mass spectra of samples with those of authentic standards. Tentative identifications were based on standard MS library data. Characteristic ion m/z (mass/charge) values used to obtain mass chromatograms are presented in Appendix 5.

5) Relative Abundance and Peak Areas of Coeluting Compounds

The relative abundance of each compound was expressed by the ratio of its total ion peak area to that of 2,4,6-trimethylpyridine (I.S.). The relative abundance of a coeluted compound was calculated by the ratio of its characteristic ion peak area to that of internal standard.

6) Statistical Analysis

The data were analyzed statistically by T-test (Steel and Torrie, 1980). Differences were considered significant when means of compared sets differed at the $p < 0.05$ level of significance.

CHAPTER IV.

RESULTS AND DISCUSSIONS

EXPERIMENT 1: Isolation of Trypsin-like Enzymes from Crawfish Hepatopancreas and Their Physicochemical Properties

A. Optimum Reaction Conditions for Proteolytic Activity

1) Effect of pH on Proteolytic Activity

The effect of pH on the hydrolysis of casein or hemoglobin was measured by incubating crude extract from crawfish hepatopancreas over the pH range of pH 1.8 to 11.0 at 45°C for 20 min. Because of precipitation of casein in the acidic pH range, hemoglobin was used as a substrate when examining proteolytic activity in the range of pH 1.8 to 5.0, whereas, casein was used in the range from pH 5.0 to 11.0. As shown in Fig. IV-1, the crude enzyme had very broad optimum activity in the pH range of 5.8 to 9.0. This pH profile established that one or more enzymes existed in the crude extract with proteolytic activity in the neutral to alkaline pH range. Additionally, proteolytic activity in the acid range was detected, although it was low compared with that at neutral and alkaline pH. The proteolytic activity near the ultimate post-mortem pH of crawfish (6.8) was within the optimum pH range. Therefore, it was decided to concentrate on the purification of enzymes with caseinolytic activity in the neutral to alkaline pH range. The determination of proteolytic

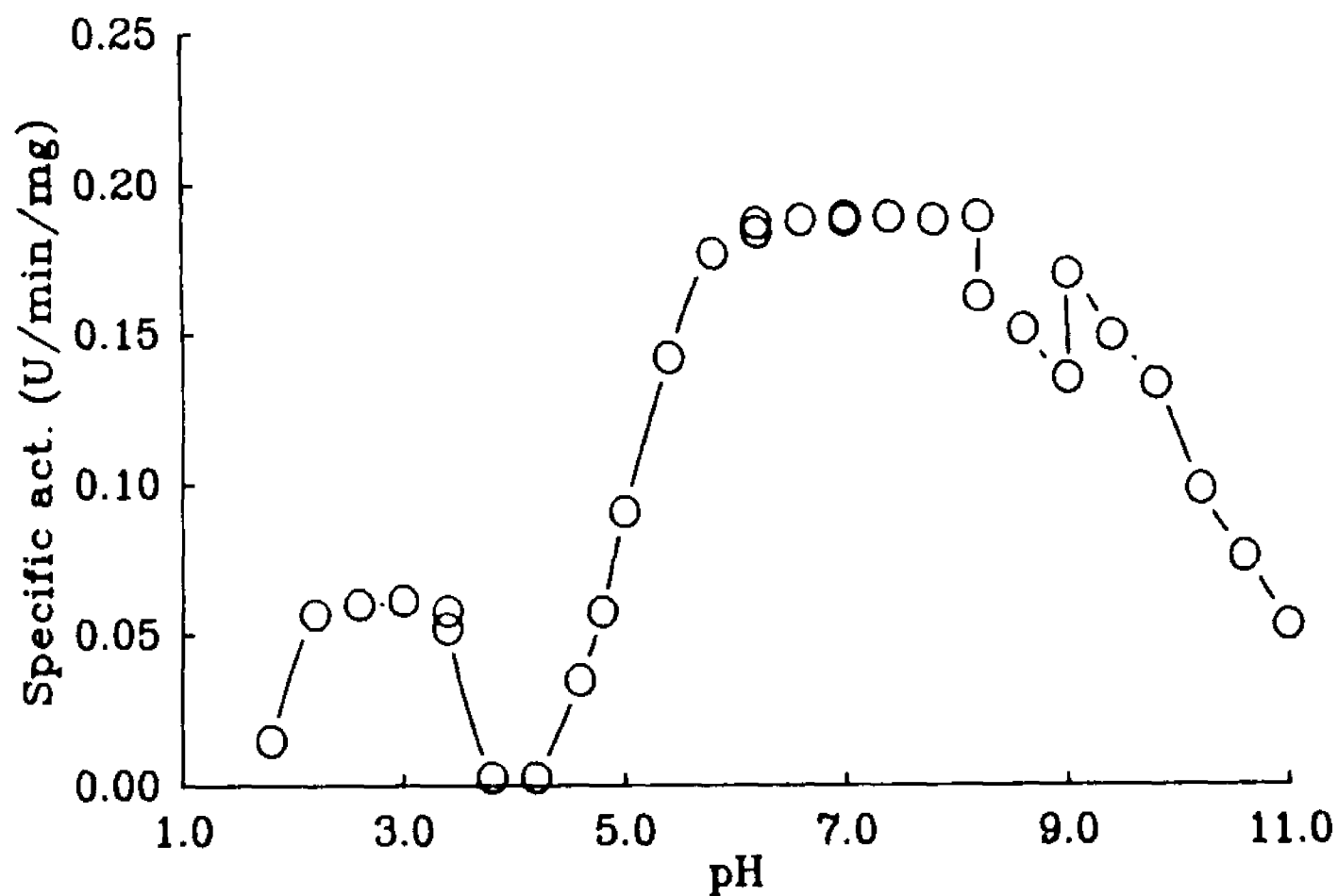


Fig. IV-1. Effect of pH on hydrolysis of casein by crude extract from crawfish hepatopancreas. Buffers used were 0.2 M glycine-0.1 M HCl (pH 1.8-3.2), 0.1 M citrate-sodium citrate (pH 3.2- 6.0), 0.1 M Na_2HPO_4 - NaH_2PO_4 (pH 6.0-8.0), 0.1 M Tris-HCl (pH 8.0-9.0), and 0.1 M Na_2CO_3 - NaHCO_3 (pH 9.0-11.0).

activity during the purification experiments was performed at pH 6.8, the actual pH of crawfish hepatopancreas.

These results were in agreement with reports by Osnes et al. (1986) who studied the release of tyrosine equivalents by krill homogenates, and several other investigators who examined crude extract from krill (Chen et al., 1978a; Konakaya, 1980; Kimoto et al., 1981).

Maximum proteolytic activity from digestive extract of five species of crab (Galgani and Nagayama, 1987a) and lobster (Galgani and Nagayama, 1987b) was obtained at pH 7.5. However, proteinases from the digestive gland of shrimp had maximal proteolytic activities against casein at pH 8.5-9 for Penaeus kerathurus and at pH 9-9.5 for Penaeus japonicus (Galgani et al., 1984), which is moderately higher than other crustacea. Digestive protease from crustacea had a broader optimum pH range than those from teleost fish or mammals. On the other hand, the crude enzyme extracted from the digestive tract of marine gastropods (Cho et al., 1983) and marine mollusca (Pyeun et al., 1983) showed distinctive proteolytic activity at acid, neutral, and alkaline pH. Crude extract from the intestine of stomachless bonefish had proteolytic activity at acid as well as alkaline pH (Jany, 1976). Thus crustacea, gastropods, mollusca, and lower teleost fish, which have no distinctive intestine structure, possibly contain acid protease in the hepatopancreas or intestine.

The structure of the intestine in teleost fish is

generally similar to that in mammalia even though there are important differences in the digestive system. The proteolytic enzymes in the internal organs of fish are distributed similarly to those of mammalia, i.e., pepsin-like in stomach (Noda and Murakami, 1981; Gildberg and Raa, 1983; Arunchalam and Haard, 1985; Squires et al., 1986), and trypsin- and chymotrypsin-like in intestine, pancreas and pyloric caeca (Yoshinaka et al., 1981; Uchida et al., 1984; Heu et al., 1991). The effect of pH on the activity of proteases from fish is very similar to that of mammalia. It has been demonstrated that the proteinases distributed in alimentary canal, pancreas, and pyloric caeca of mackerel, Scomber japonicus, and sardine, Sardinops melanosticta, were active in alkaline pH range, but those in stomach had higher activity at acidic pH (Kim et al., 1986).

2) Effect of Temperature on Caseinolytic Activity

The optimum temperature for caseinolytic activity of the crude enzyme solution from crawfish hepatopancreas was found to be 50°C, with total loss of enzyme activity occurring at 75°C (Fig. IV-2). Activity at 25°C was approximately 35% of the maximum activity, which is considered to be high proteolytic activity at physiological temperature.

Proteases from the digestive extract of two shrimp Penaeus kerathurus and Penaeus japonicus had optimum temperature for proteolytic activities at 50°C (Galgani et

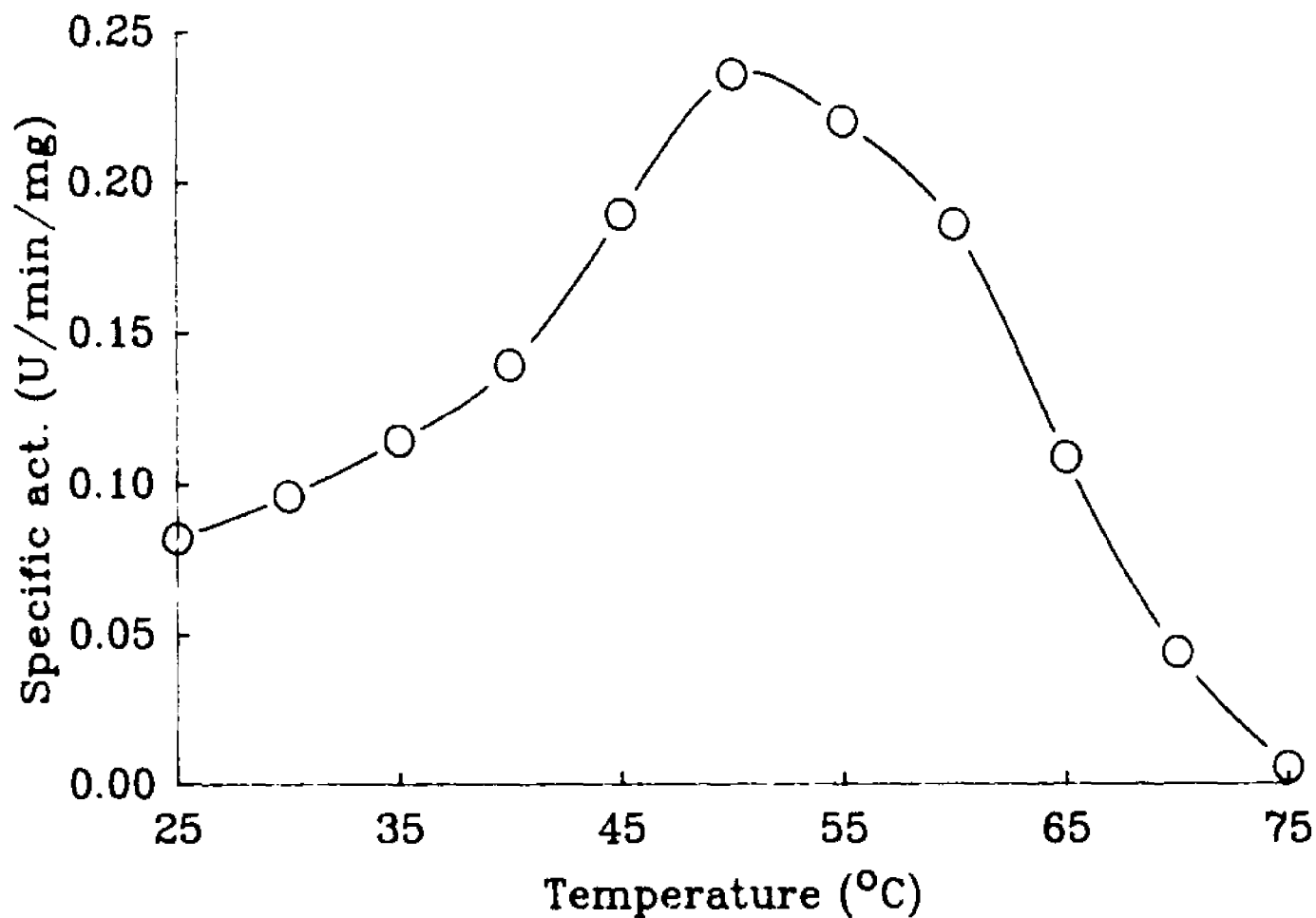


Fig. IV-2. Effect of temperature on hydrolysis of casein by crude extract from crawfish hepatopancreas. Reaction was performed at pH 6.8 (0.1 M Na_2HPO_4 - NaH_2PO_4) for 20 min at indicated temperature.

al., 1984) and in the temperature range of 45 to 55°C for five species of crab (Galgani and Nagayama, 1987a), but the digestive protease from lobster Panulirus japonicus had higher optimum temperature at 60°C (Galgani and Nagayama, 1987b). Also, caseinolytic activity of the crude extracts from krill, shrimp, and mysis was maximum at 50°C, 55°C and 45°C, respectively (Konagaya, 1980). Proteolytic enzymes active in neutral and alkaline pH range from the digestive tract of marine gastropods had optimum temperature between 45-55°C, but an extremely low optimum temperature was detected at 30°C in sea hare (Cho et al., 1983).

The maximum enzymatic activities in muscle were found at 60-65°C, while those in internal organs were at 45-50°C. These observations were based on optimum caseinolytic activities distributed in the white muscle of 4 species of fresh water fish, 21 species of marine fish, 2 species of mammals, and the internal organs of carp (Iwata et al., 1974). The optimum caseinolytic activities of alkaline proteinases from alimentary canal, pyloric caeca, and pancreas of mackerel and sardine were found to be 45-50°C (Kim et al., 1986). Also, optimum activity of alkaline proteinases from the muscle of file fish, hag fish, and cat shark were at higher temperatures than those from their internal organs (Nam and Pyeun, 1983). These studies suggest that maximum proteolytic activity of digestive proteolytic enzymes from fish had lower temperature optima than those in muscle.

3) Activation Time for Caseinolytic Activity

Crude enzyme from crawfish hepatopancreas was incubated at 40°C to determine activation time for proteolytic activity. Residual caseinolytic activity after different intervals of incubation was determined. Caseinolytic activity decreased linearly with prolonged incubation time (Fig. IV-3) resulting in 25% decrease of original activity after 60 min incubation.

Generally, trypsin is distributed as trypsinogen in mammalia (Figarella et al., 1975; Brodrick et al., 1978) and fish (Simpson and Haard, 1984a; de Haen et al., 1977; Reeck and Neurath, 1972), but there is no evidence of a zymogen form in crustacea (Vonk and Western, 1984). The results of the present experiment suggests the possibility that the proteases in crawfish hepatopancreas either do not exist as zymogen type enzymes or autolyze rapidly during activation. Another possibility is inhibition by protein hydrolysate of other proteins during activation because the blank test increased with extended incubation time.

4) Effect of Ca^{2+} Ions on Caseinolytic Activity

Crude enzyme from crawfish hepatopancreas was incubated with various concentration of Ca^{2+} ions to determine the effect of Ca^{2+} ions on caseinolytic activity. As shown in Fig IV-4, caseinolytic activity was highest at concentrations of 0.025 to 0.05 mM Ca^{2+} ions. Caseinolytic activity increased approximately 15% in the presence of Ca^{2+} compared to without

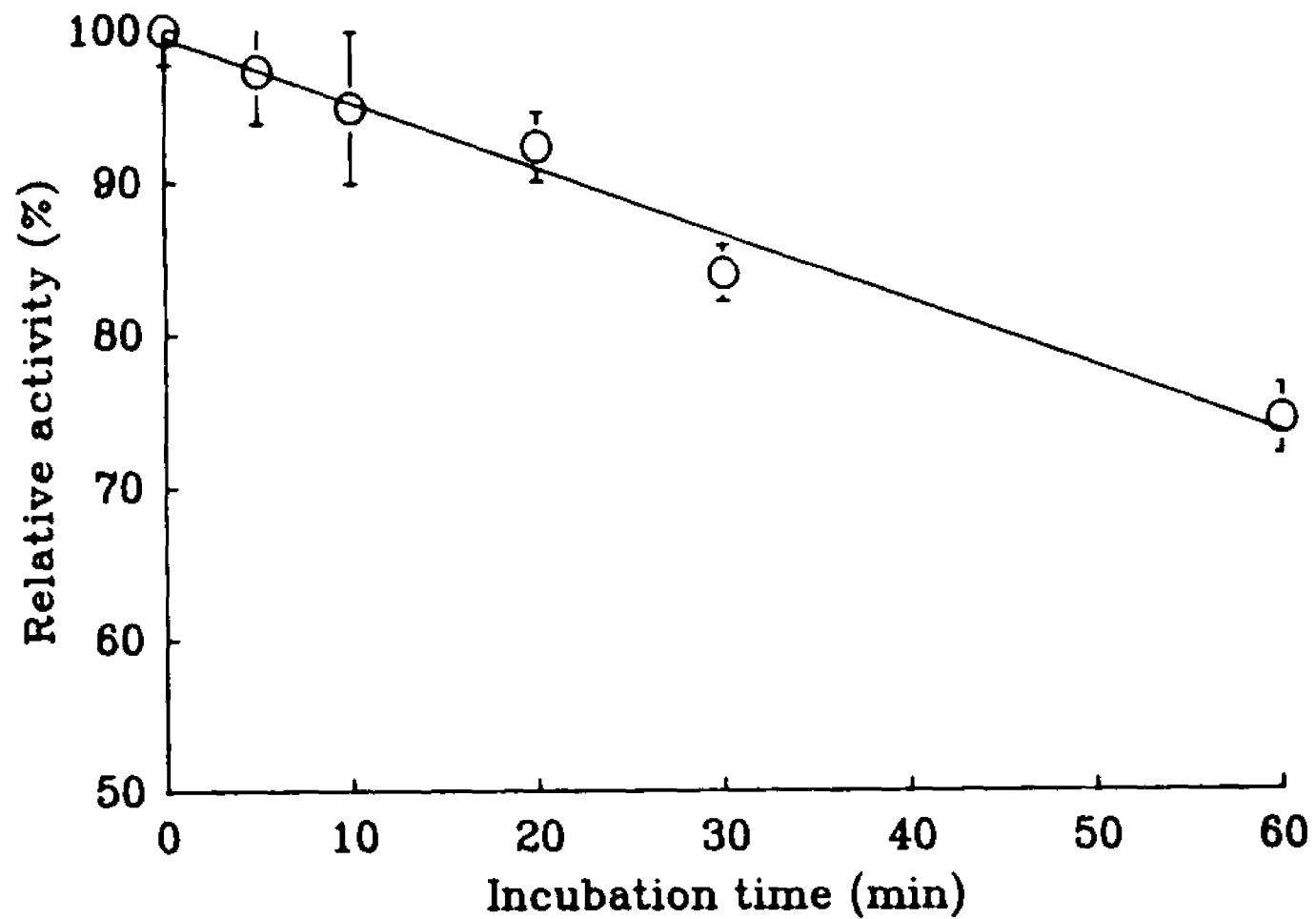


Fig. IV-3. Effect of incubation time on caseinolytic activity of crude extract from crawfish hepatopancreas. Reaction was performed at pH 6.8 and 45°C for 20 min.

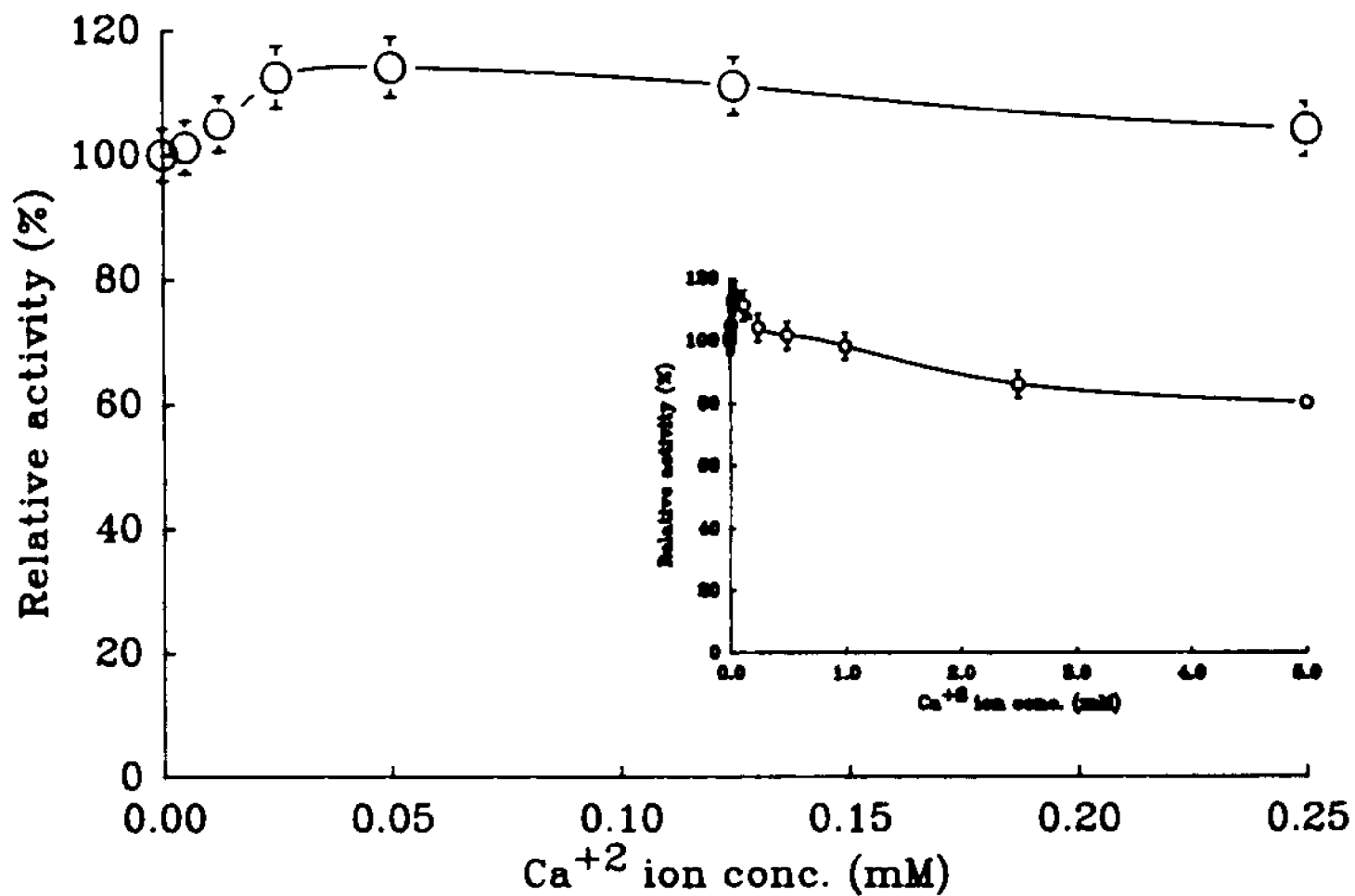


Fig. IV-4. Effect of Ca^{+2} ions on caseinolytic activity of crude extract from crawfish hepatopancreas. Reaction conditions were the same as in Fig. IV-3.

Ca^{2+} ions. Caseinolytic activity did not change with a concentration of 1 mM Ca^{2+} ; however, the activity decreased about 20% from original activity at 5 mM of Ca^{2+} as indicated in insert (Fig. IV-4).

The inclusion of Ca^{2+} ions up to a concentration of 0.25 mM increased the proteolytic activity of crawfish hepatopancreatic enzymes. This agrees with results reported for digestive proteinases from crab (Galgani and Nagayama, 1987a; 1988). However, calcium ions did not affect the tryptic activity of shrimp (Gates and Travis, 1969), and inhibited trypsin activities in Atlantic blue crab (Dendinger and O'Connor, 1990) and crayfish (Zwilling et al., 1969).

B. Purification of Trypsins from the Hepatopancreas

All the samples for assay of enzyme activity and protein concentration during the purification steps were dialyzed against distilled water to exclude the possible disrupting effect of ions in the enzyme reaction and to remove possible interference in the protein assay.

Two hundred and seventeen g of hepatopancreas of crawfish were homogenized and extracted with 2 volume of 1% NaCl containing 1 mM 2Na-EDTA. After centrifugation, the crude extract was immediately subjected to 30-60% acetone fractionation. A brownish black protein which precipitated

in the centrifuge tubes was dissolved in cold 0.02 M Tris-HCl buffer (pH 6.8) containing 0.5 M NaCl, 5 mM CaCl₂, and 1 mM benzamidine, and then dialyzed against the same solution for benzamidine Sepharose 6B affinity chromatography. During dialysis of the acetone fraction, an unidentified yellow pigment diffused into the buffer. Also, the sample in dialysis tubing turned to a black color with increasing dialysis time, which was presumed to be due to polyphenol oxidase activity. The reddish black color sample was loaded on a benzamidine Sepharose 6B affinity column, which is selective for trypsin-like enzymes.

After affinity chromatography, the total tryptic activity was nearly the same as the acetone fraction. A slight loss (11%) of tryptic activity occurred during 2 days of dialysis. The benzamidine Sepharose 6B fraction (80 mg protein) after dialysis for two days against 0.02 M Tris-HCl buffer (pH 6.8) was applied on an initial DEAE-Sephacel column (3 x 35 cm) and eluted with a 2,000 ml linear gradient ranging from 0 to 2.0 M NaCl. With this ionic exchange chromatography, four kinds of trypsins were eluted in fraction numbers of 74-94 for trypsin A and B, 96-112 for trypsin C, and 118-136 for trypsin D (Fig. IV-5). These enzymes were tentatively named as trypsin A, B, C, and D using the elution order obtained in the first ionic exchange chromatography. Four species of trypsin-like enzymes

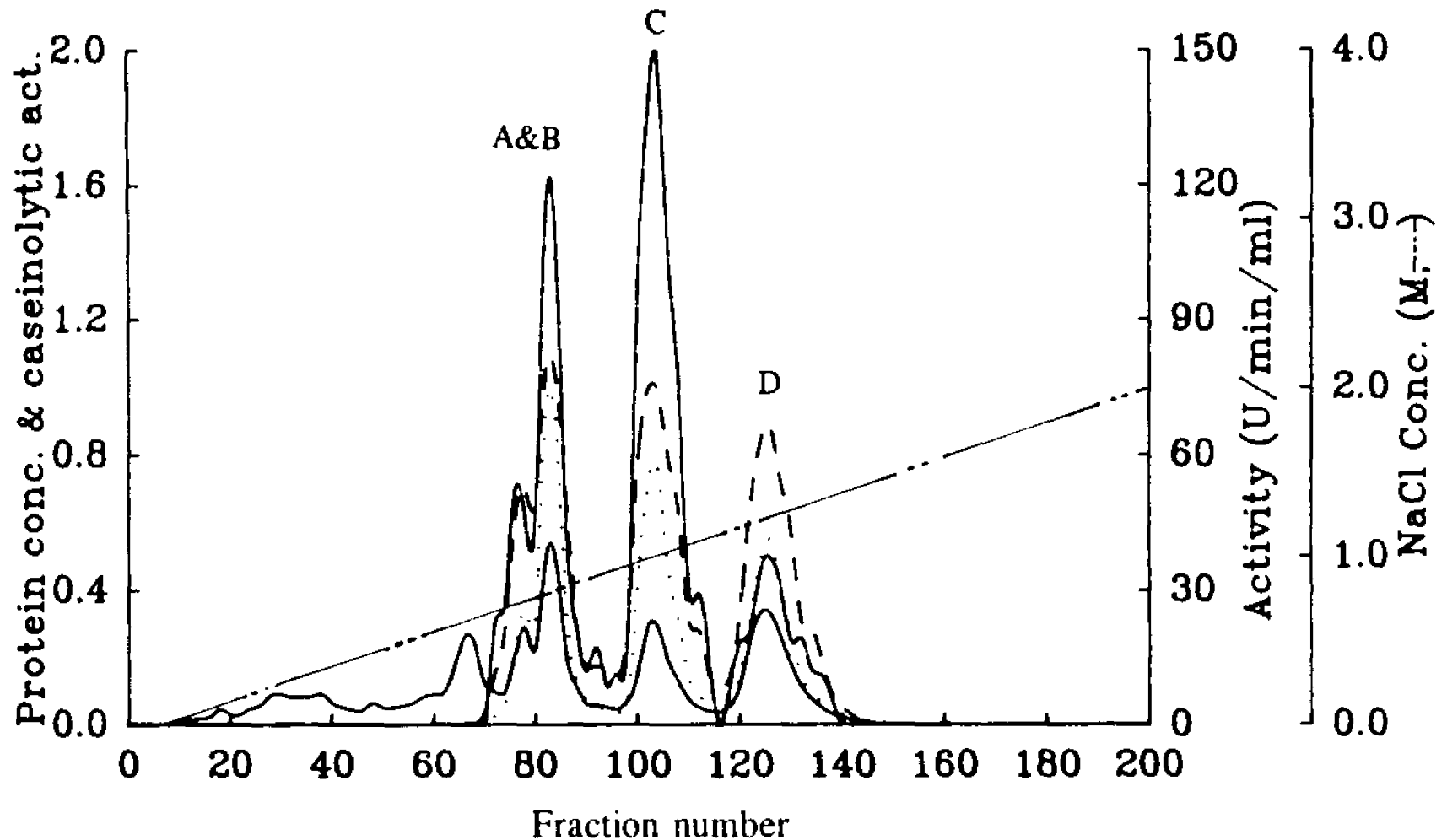


Fig. IV-5. Chromatogram of a 1st DEAE-Sephacel ionic exchange chromatography of 30-60% acetone fraction. Flow rate was 40 ml/hr and fraction volume 10 ml per tube. The symbols were as follows: —, protein concentration at 280 nm; ·····, proteolytic activity; ---, esterolytic activity; — — —, amidolytic activity ($\times 10^3$), and ·····, salt concentration.

were separated in the high concentration range of NaCl, which suggests that these enzymes were very strong anionic trypsin-like enzymes.

After first DEAE-Sephacel A-50 chromatography, the concentration process was ineffective. Concentration with ultrafiltration caused about 15% loss of enzyme activity due to membrane passage and slow filtration. Acetone precipitation also was ineffective as a way to concentrate these samples, which were dissolved in high salt solution. This phenomenon might be peculiar to these enzyme fractions dissolved in high concentration of salt. Thus, enzyme concentration was performed after 2 days of dialysis against D.W, which diminished salt concentration, and allowed subsequent ultrafiltration. As a result of this tedious procedure, approximately half of trypsin was lost during dialysis and ultrafiltration.

As shown in Fig. IV-5, trypsin A and B were not completely separated by first ionic exchange chromatography, so their combined fraction (Fraction number 74-94) was dialyzed against 0.02 M Tris-HCl for 2 days to remove salts and then rechromatographed on the DEAE-Sephacel (2 x 20 cm) with a 1,000 ml linear gradient ranging from 0.5 to 1.5 M sodium chloride. With this rechromatographic step, trypsin A and B were completely separated (Fig. IV-6).

Each separated fraction was concentrated with ultrafiltration and dialyzed against 0.02 M Tris-HCl

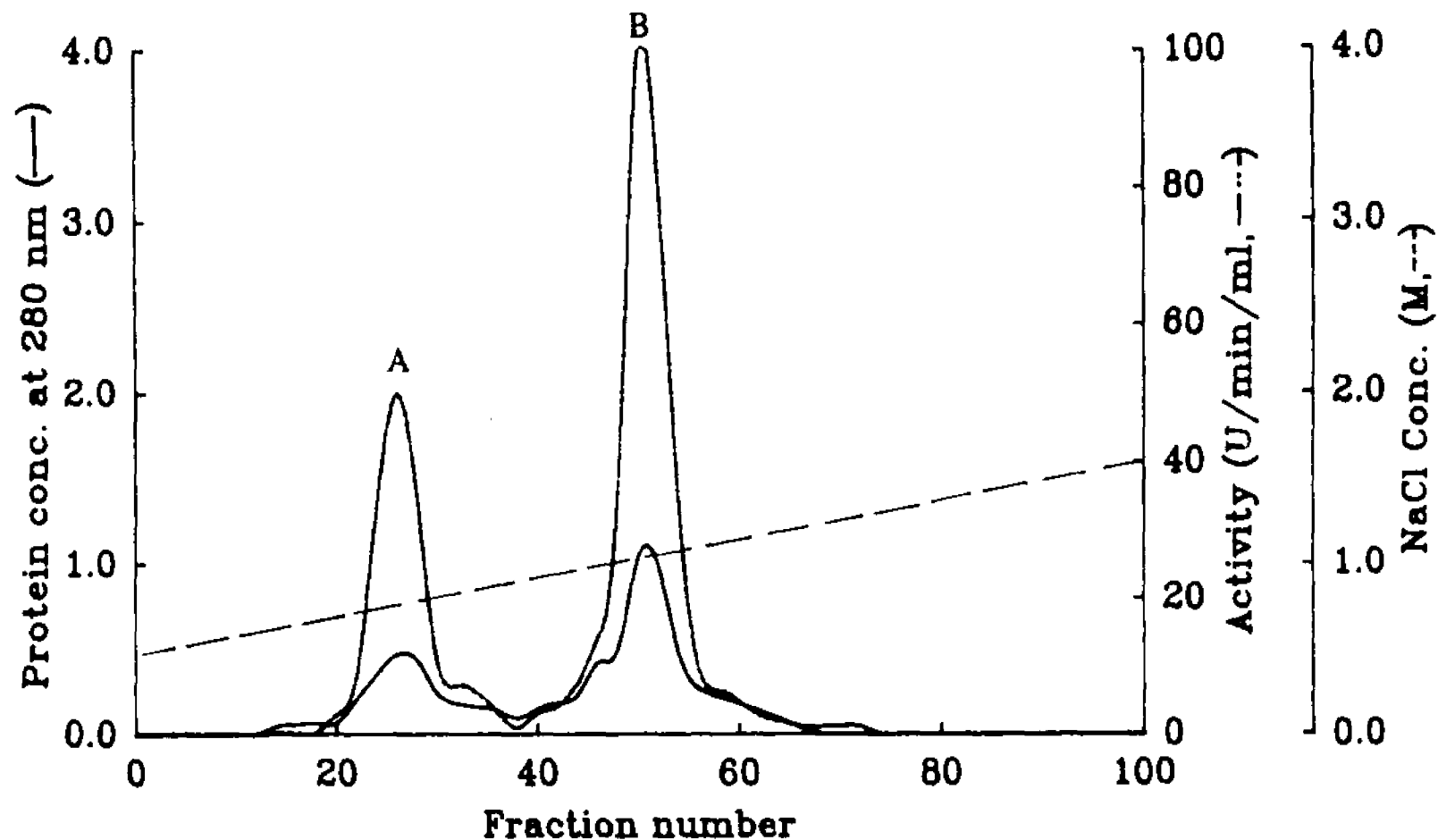


Fig. IV-6. Chromatogram of a 2nd DEAE-Sephacel ionic exchange chromatography of A and B fraction from the 1st DEAE-Sephacel chromatography. Chromatographic condition was the same as in Fig. IV-5. The symbols were as follows: —, protein concentration at 280 nm; -·-·-, esterolytic activity, and ---, salt concentration.

containing 0.1 M NaCl for Sephacryl S-200 gel filtration. After gel filtration (Fig. IV-7), trypsin A (96-105), B (88-98), C (94-104), and D (88-96) were concentrated with acetone precipitation. Acetone-precipitated fractions were dissolved in the above buffer and dialyzed against D.W. With this final gel filtration, purity of trypsin A, B, C, and D was increased 56, 100, 87, and 64 fold, respectively. This purity was higher than that found with trypsin-like enzymes in the intestine of shrimp (Gates and Travis, 1969), cod (Raae and Walther, 1989; Asgeirsson et al., 1989; Simpson and Haard, 1990), carp (Cohen et al., 1981a), anchovy (Martinez et al., 1988), and starfish (Camacho et al., 1970). However, trypsins from krill (Kimoto et al., 1983) and Atlantic blue crab (Dendinger and O'Connor, 1990) were purified with remarkably high purity.

The outline and results of purification of the four trypsins based on the esterolytic activity for TAME are summarized in Table IV-1. The four trypsin-like enzymes were isolated only in small quantities by the present purification procedures. Starting with 217 g of the crawfish hepatopancreas; 2.3 mg of trypsin A, 4.4 mg of trypsin B, 3.2 mg of trypsin C, and 2.5 mg of trypsin D were obtained. The present purification procedure produced trypsins with high purity and a combined yield of 18.6%. Purified trypsins dialyzed with D.W. were divided into several 0.1 ml aliquots and stored at -20°C for use in subsequent characterization studies.

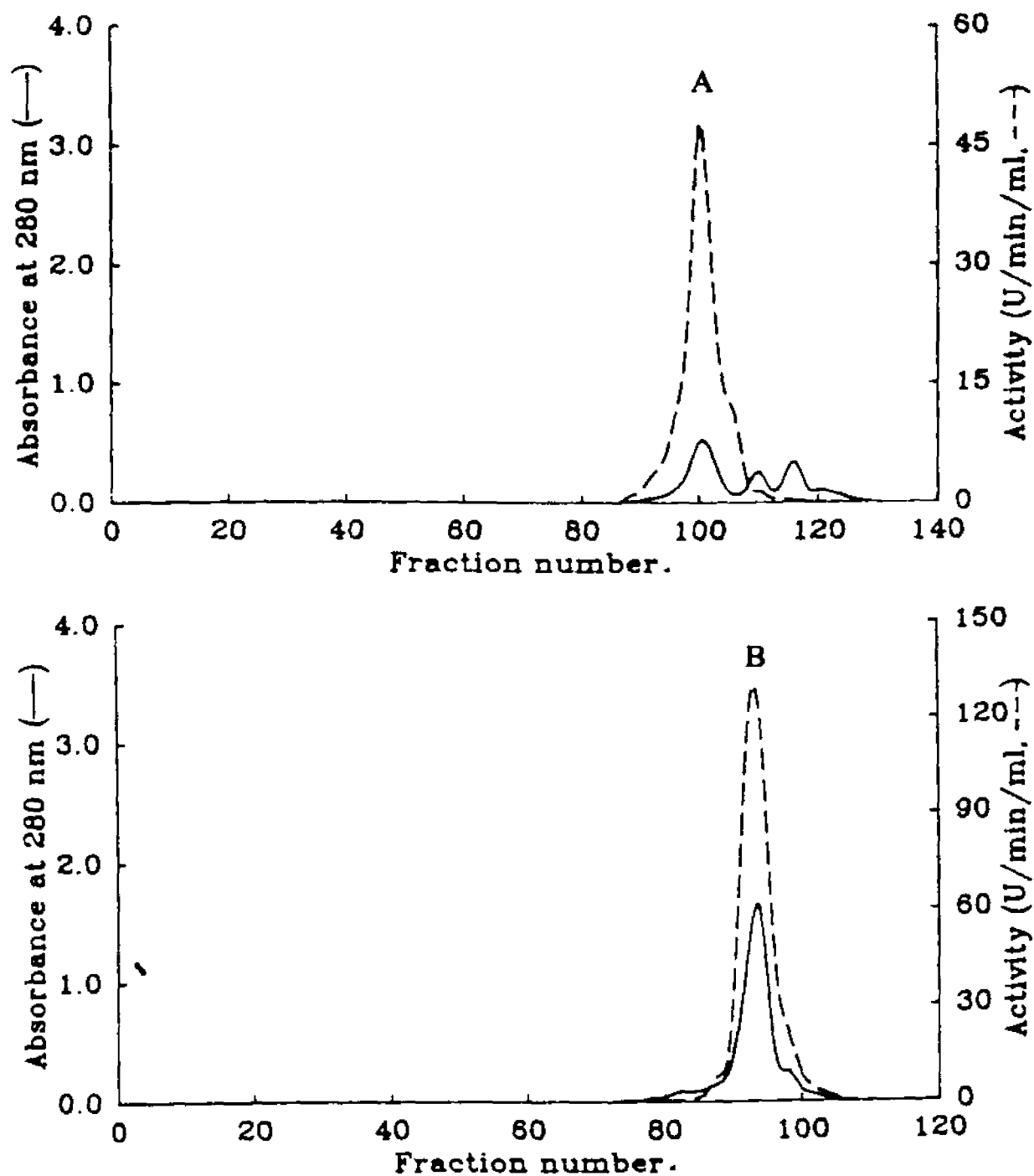


Fig. IV-7. Chromatograms of Sephacryl S-200 gel filtration from A and B from the 2'nd DEAE-Sephacel chromatography, and C and D fraction from 1'st DEAE-Sephacel chromatography. Flow rate was 10 ml/hr and fraction volume was 3.6 ml.

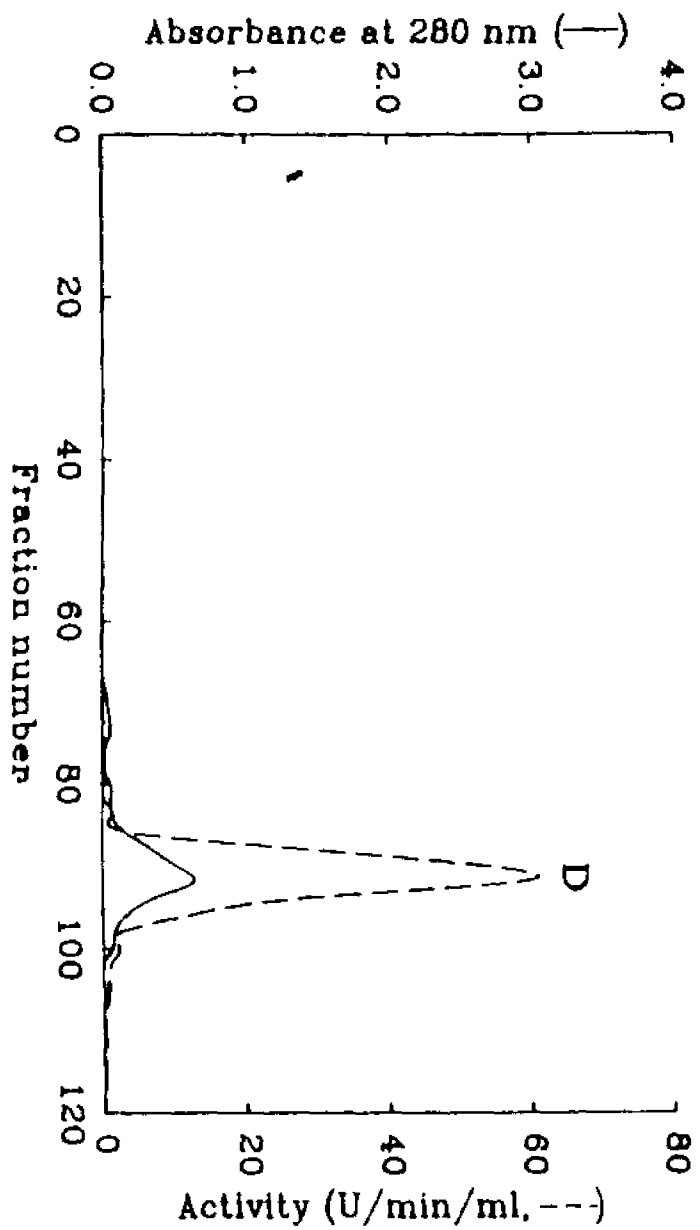
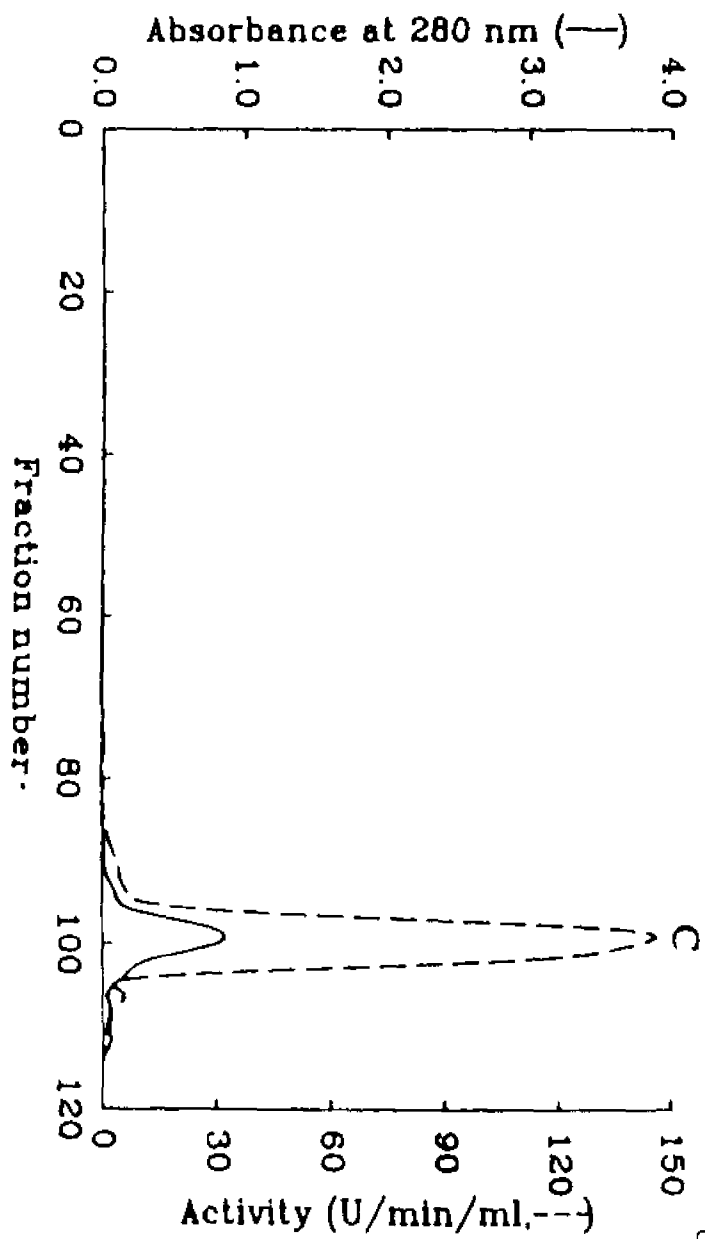


Fig. IV-7 (continued)

Table IV-1. Purification of anionic trypsins from crawfish hepatopancreas

	Total vol. (ml)	Total prot. (mg)	Total act. (U/ml)	Specif. act. (U/mg)	Fold	Yield %
Crude extract	570	5,500	32,000	5.8	1	100
Acetone fraction (30-60%)	240	1,300	27,000	21	3.7	86
Benzamidine Sepharose 6B fraction	320	79	24,000	300	38	75
1'st DEAE-Sephacel fraction						
A & B	15	29	9,800	350	60	31
C	5	6.6	3,100	460	80	10
D	5	4.8	1,600	320	56	5.0
2'nd DEAE-Sephacel fraction						
A	6	6.3	1,300	210	36	4.2
B	6	8.9	4,800	550	95	15
Sephacryl S-200 fraction						
A	2	2.3	750	320	56	2.4
B	2	4.4	2,500	570	100	8.1
C	2	3.2	1,600	500	87	5.1
D	2	2.5	940	370	64	3.0
Total	8	12.4	5,790			18.6

Crawfish hepatopancreas sample was 217 g and extracted with 2 volume of 1% NaCl containing 1 mM 2Na-EDTA. Activities were determined with 1 mM TAME as a substrate at 25°C and pH 8.1. Trypsin A + B, C and D were separated by the 1'st DEAE-Sephacryl step and A and B were separated by the 2'nd DEAE-Sephacryl step.

Yield calculated from the total initial activity.

Purification of the trypsin-like enzymes from crawfish hepatopancreas revealed that four different trypsin-like enzymes exist in crawfish hepatopancreatic tissue, possessing the ability to hydrolyze TAME, BAPNA, and casein. The chromatographic properties of these enzymes varied from the trypsin-like enzymes in teleost fish (Pyeun and Kim, 1986; Pyeun et al., 1988; Pyeun et al., 1990) previously studied by the present investigator. Yield was calculated from the total initial activity based on esterolytic activity for TAME. The separation of the proteases on the DEAE-Sephacel column was achieved by using high salt concentration, signifying that the enzymes possessed a high negative charge density on the molecular surface at the pH employed.

As shown in Fig. IV-8, numerous diverse proteins other than the trypsin-like enzymes were effectively removed with each purification step. Fraction obtained with benzamidine Sepharose 6B presented one major band with only a few minor bands, which indicates highly effective purification steps for the separation of trypsin-like enzymes. The migration of each protease was similar to bromophenol blue dye because isoelectric points of proteases were very low. Thus, final purity was demonstrated with the combination of gel chromatograms and isoelectrofocusing.

Purified enzymes were subjected to disc-PAGE. One gel of each enzyme was stained, while another gel was sliced into 3 mm segments. Each trypsin was extracted from the unstained gel

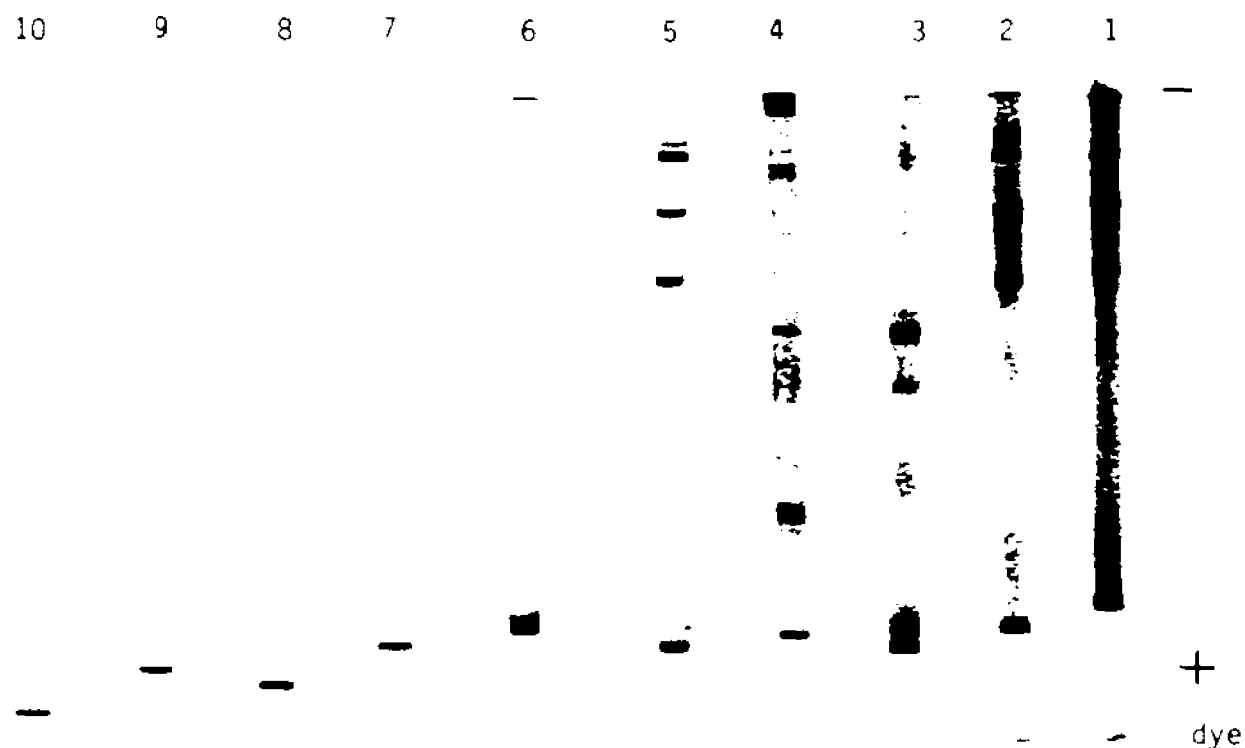


Fig. IV-8. Discontinuous polyacrylamide gel electrophoresis (10%) throughout purification steps. Lane 1, crude enzyme; 2, 30-60% acetone fraction; 3, benzamidine Sepharose 6B fraction; 4, nonabsorbed benzamidine Sepharose 6B fraction; 5, nonabsorbed benzamidine Sepharose 6B fraction (7.5%); 6, A and B fraction from 1'st DEAE-Sepharcel ionic exchange chromatography; 7, purified trypsin A; 8, purified trypsin B; 9, purified trypsin C, and 10, purified trypsin D after gel filtration.

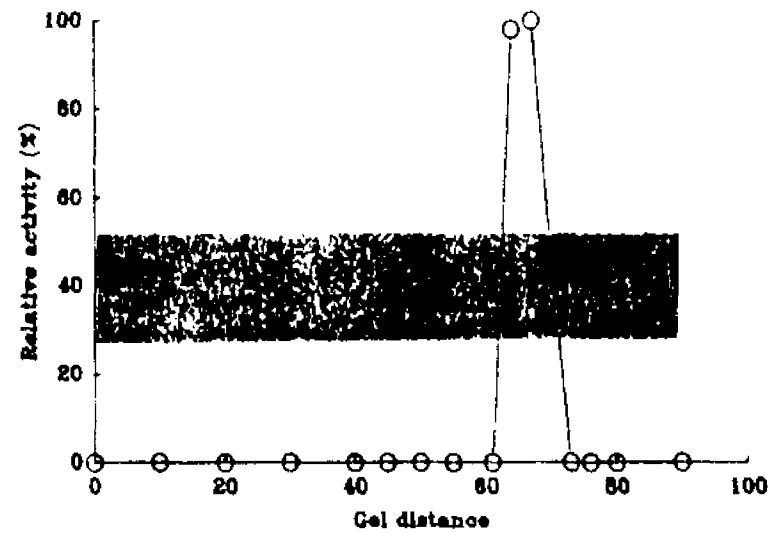
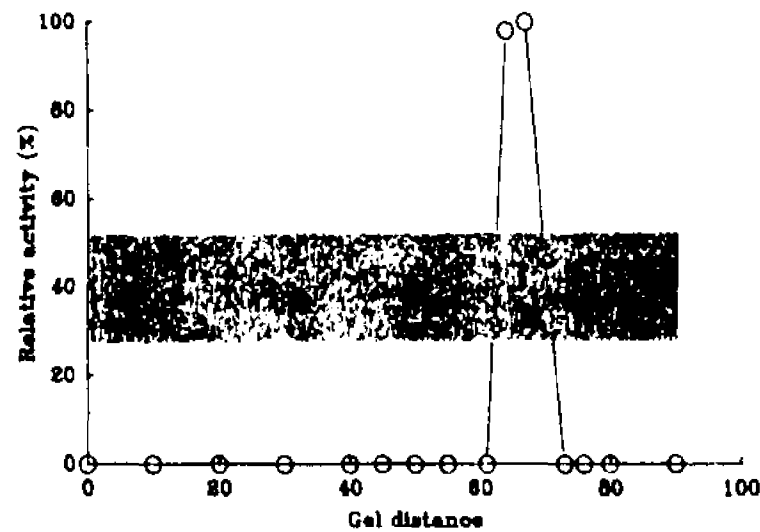
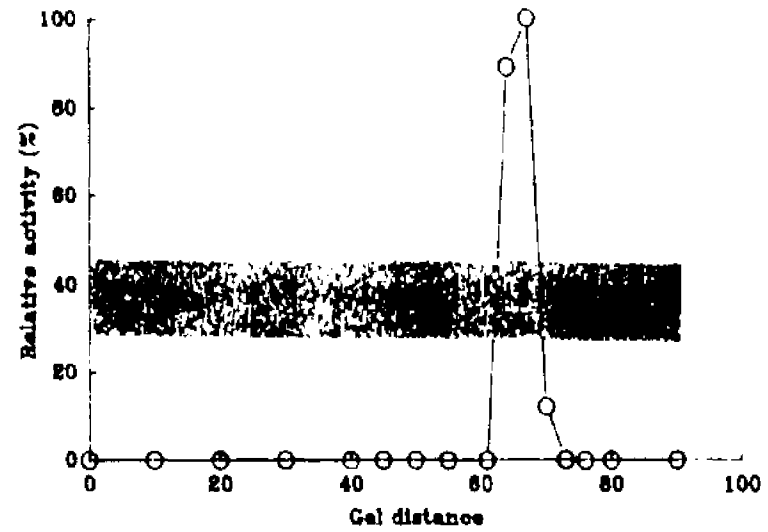
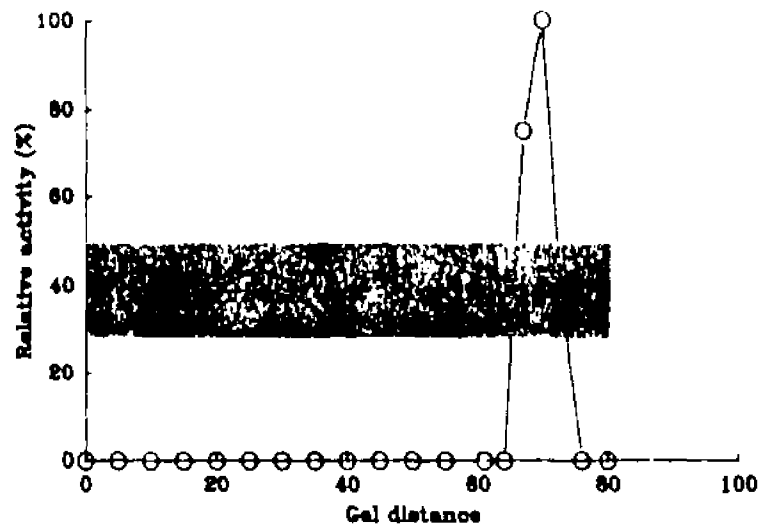


Fig. IV-9. Discontinuous polyacrylamide gel electrophoresis of four purified protease. Graphs demonstrate the extracted enzyme from the unstained gels. Section number indicates the number of 3 mm thick gel slices.

1988), trypsin from Streptomyces griseus (39,600/M.cm) (Olafson and Smillie, 1975), and trypsin from carp pancreas (56,200/M.cm) (Cohen et al., 1981a).

2) Determination of Molecular Weight

The molecular weights of the four trypsin-like enzymes were estimated by Sephacryl S-200 gel filtration (Fig. IV-10). The molecular weights (M.W.) of trypsin A, B, C, and D were calculated at 23,800, 27,900, 24,800, and 31,400 D, respectively, using bovine serum albumin (66,000), carbonic anhydrase (29,000), cytochrome C (12,400), and aprotinin (6,500) as standards. The molecular weights of trypsin A, B, and C were similar to those of mammalian and fish trypsin, but trypsin D had a slightly higher M.W. than that reported for other trypsins as well as other trypsins in this study.

According to Keil (1971) trypsin enzymes have a M.W. around 20,000 to 25,000 D. However, trypsins from marine organisms, either invertebrates or vertebrates, in most cases have been found to have M.W. in the range of 24,000-30,000 D. The M.W. of trypsin-like enzymes from crustacean also vary widely. A trypsin-like enzyme from hepatopancreas of white shrimp Penaeus setiferus was estimated to have M.W. of 24,000 D (Gates and Travis, 1969). A similar M.W. was reported for one trypsin-like enzyme from digestive glands of the Japanese spiny lobster Panulirus japonicus, (Galgani and Nagayama, 1987b). The M.W. of trypsin-like enzymes by gel filtration

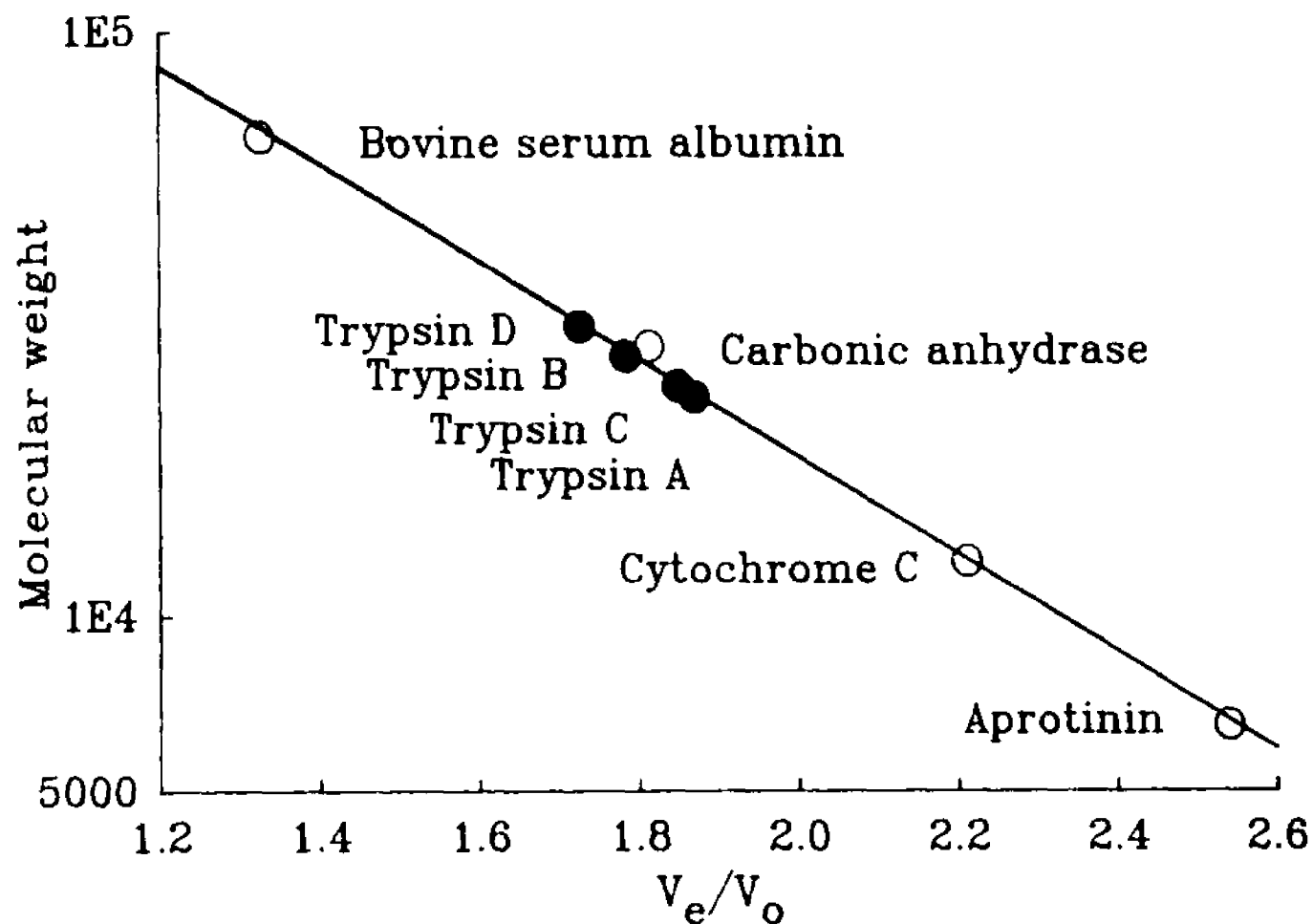


Fig. IV-10. Determination of molecular weights of crawfish hepatopancreatic proteases by Sephacryl S-200 gel filtration. The chromatographic condition was the same as in Fig IV-7.

were reported to be 25,000 in gastric juice of lobster (Brockerhoff et al., 1970) and digestive gland of shrimp (Galgani et al., 1984), but the M.W. of protease in the hepatopancreas of jonah crab and rock crab were evaluated to be 16,700 and 20,500, respectively, by Sephadex G-100 (Brun and Wojtowicz, 1976). A somewhat higher M.W. has been found for three trypsins from crab Eriocheir japonicus of approximately 29,000 D, and the enzymes did not dissociate into subunits in the presence of SDS (Muramatsu and Morita, 1987). In other work, trypsins from crustacea were found to have higher M.W. i.e., 33,500 from the midgut gland of crab Callinectes sapidus (Dendinger and O'Connor, 1990) and 30,000 to 31,000 from antarctic krill (Osnes and Mohr, 1985b).

Uncharacteristically low M.W. (11,000-13,000) proteases, which have similar enzymatic properties to trypsin, have been identified in the digestive organ of decapods. These may be a member of an unknown family of proteases which have not been reported in vertebrates (Zwilling and Neurath, 1981). The enzymes may act in a compensatory manner due to the apparent absence of peptidic and chymotryptic endopeptidases in crustacea (DeVillez, 1975). They have been observed in the digestive organs of crustacea such as lobster (Galgani and Nagayama, 1987b; Brockerhoff et al., 1970), shrimp Penaeus spp (Galgani et al., 1984), and crayfish Astacus fluviatilis (Pfleiderer et al., 1967) and Orconectes virilis (DeVillez and Lau, 1970). Molecular weights of trypsin in teleost fish

are similar to that of mammals. In the intestine of fish and mammals, a M.W. around 20,000-25,000 D has been reported for trypsins from African lungfish (Reeck and Neurath, 1972), sardine (Murakami and Noda, 1981), carp (Cohen et al., 1981a), capelin (Hjelmeland and Raa, 1982), Greenland cod (Simpson and Haard, 1984a), mackerel (Kim and Pyeun, 1986), skipjack (Pyeun et al., 1988), Atlantic cod (Asgeirsson et al., 1989; Simpson et al., 1990), and menhaden (Pyeun et al., 1990).

3) Determination of Amino Acid Composition

The amino acid composition of purified trypsin A, B, C, and D compared to the analogous enzymes of crayfish Astacus leptodactylous, shrimp, krill, and bovine are given in Table IV-2. The amino acid residues presented in integral numbers, were computed from the best fit to their molecular weights, without tryptophan and cysteine, as determined from gel filtration. Except for the content of glycine and alanine, similar amino acid compositions were clearly observed among crawfish (Procambarus clarkii) trypsins as well as the trypsin from crayfish Astacus leptodactylous (Zwilling and Tomasek, 1970). The compositions were similar to the trypsin from krill except for leucine, alanine, isoleucine, and threonine, and to trypsin from shrimp except for glycine, threonine, alanine, and leucine. Crawfish trypsins were rich in alanine, glycine, and potential acidic amino acid residues but low in methionine, phenylalanine, and basic amino acids. These

Table IV-2. Amino acid compositions of crawfish hepatopancreatic trypsin. The values are given in residues per molecule

A.A	Crawfish				Crayfish ¹	Shrimp ²	Krill ³	Bovine ⁴
	A	B	C	D				
Asp	31	40	43	46	30	30	26	22
Glu	19	27	16	28	21	24	29	14
Ser	16	19	18	21	17	24	24	33
Gly	45	55	44	59	28	28	44	25
His	3	4	4	7	5	5	5	3
Arg	2	3	3	2	2	3	2	2
Thr	14	16	16	20	15	10	11	10
Ala	28	27	25	29	16	16	19	14
Pro	15	15	11	17	10	11	13	9
Tyr	8	10	9	11	11	10	10	10
Val	19	19	17	22	18	18	17	17
Met	1	1	2	3	2	2	3	2
Ile	14	16	13	18	15	14	11	15
Leu	18	19	16	19	16	10	12	14
Phe	7	7	6	6	7	6	5	3
Lys	3	4	4	5	3	5	4	14
Ratio of acidic to basic amino acids								
	6.0	6.5	5.5	5.3	4.3	4.2	5.0	1.9
No of residues								
	243	280	247	313	218	216	247	207

¹ Zwilling and Tomasek, 1970. Nature, 228; 57

² Gates and Travis, 1969. Biochem. 8; 4483

³ Kimoto et al., 1983. Agric. Biol. Chem. 47: 529

⁴ Walsh and Neurath, 1964. Proc. Nat. Acad. Sci., 52; 884.

results are consistent with anionic properties of other trypsin-like enzymes from fish.

As presented in Table IV-2, trypsins from crustacea have similar ratios of acidic to basic amino acids in the range of 4.2 to 6.0. This was quite different from mammalian trypsin, which have basic isoelectric points. Of particular interest, lysine content in bovine trypsin is significantly higher than in crustacean trypsins. The high ratio of acidic to basic amino acid residues supports the observation that trypsin-like enzymes from crawfish have low isoelectric points and that the enzymes were bound strongly to the anionic exchange resin and eluted in a high concentration of salt solution. From binding of the enzymes to the anionic exchange column (Fig. IV-5), it appears that the enzymes have an increasing negative surface charge at neutral pH in the order trypsin D > C > B > A. This corresponds with a decrease of aspartic acid residues from trypsin D to trypsin A.

The higher ratio of acidic to basic amino acid residues gives the molecule a net negative charge and tends to decrease its solubility at acidic pH. This could result in an irreversible conformational change causing inactivation of the enzyme at low pH. Trypsins from crustacea and krill had a total of 5-8 arginine and lysine residues per molecule in comparison to 16 residues for bovine trypsin. Thus, crustacean trypsins were possibly more stable at neutral pH against autolysis than is bovine trypsin.

The similarities in amino acid compositions among the trypsins reflect a considerable measure of homology in primary structure (Prahl and Neurath, 1966; Zwilling et al., 1975). From the amino acid composition of the 4 crawfish trypsins, it may be concluded that they are similar in primary structure and are like other anionic trypsins derived from fish. Further analysis of cysteine and tryptophan is needed to obtain complete amino acid composition of trypsins.

4) Determination of Isoelectric Point

Isoelectrofocusing resulted in a single protein band (Fig. IV-11). The trypsins from crawfish hepatopancreas were identified as strong anionic proteins with an isoelectric point of less than 3.0. The result was consistent with the large number of acidic amino acid residues that proteases contain.

Isoelectric points of trypsin-like enzymes from the crustacean digestive enzyme were reported to be very low, i.e., 4.0 for the Atlantic blue crab Callinectes sapidus (Dendinger and O'Connor, 1990), 3.8 for crayfish (Zwilling et al., 1969), 2.6 for Arctic krill (Kimoto et al., 1983), and less than 3.5 for Antarctic krill (Osnes and Mohr, 1985b). This contrasts with bovine trypsin which has a high isoelectric point between 9.3 and 10.1 (Walsh, 1970). Also the low molecular weight protease purified from crayfish Astacus fluviatilis is distinguished from most of the accompanying

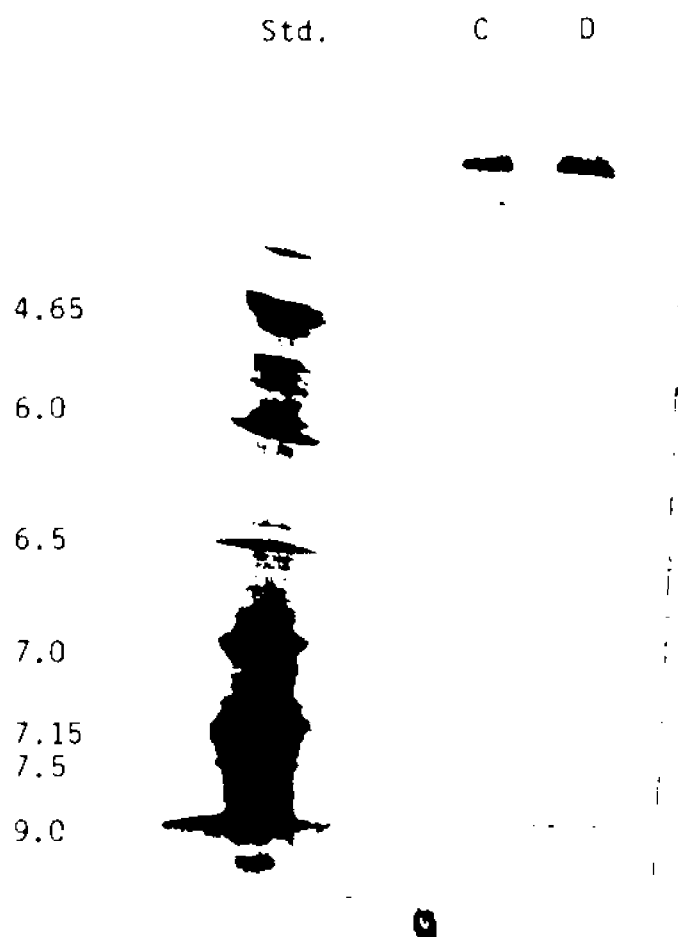


Fig. IV-11. Isoelectrofocusing of purified crawfish hepatopancreatic trypsin C and D. Std., pH standard; C, trypsin C, and D, trypsin D.

proteins by its small size as well as low isoelectric point (Zwilling and Neurath, 1981). Anionic trypsins seem to be quite common in marine organisms, and have been described in starfish (Winter and Neurath, 1970; Kozlovskaya and Elyakova, 1974), capelin (Hjelmeland and Raa, 1982), catfish (Yoshinaka et al., 1984), anchovy (Martinez et al., 1988), and salmon (Pivnenko et al., 1990).

EXPERIMENT 2. Enzymatic Characterisation of Purified Trypsins from Crawfish Hepatopancreas

A. Effect of pH on Trypsin Activity

1). Effect of pH on Proteolytic Activity

The caseinolytic activities of the four trypsins were maximum in the broad pH range of 6.0 to 9.5 (Fig. IV-12). This indicates that caseinolytic activities of trypsins were not sensitive to pH changes in neutral and alkaline pH range. Proteolytic activities of trypsin A, B, C, and D at physiological pH (6.8) were higher, with specific activities of 12, 6.6, 6.6, and 10 U, respectively. The activities of enzymes decreased below pH 5.0 and relative activities at pH 4.0 compared to their maximum activities were found to be 13% for trypsin A, 7% for trypsin B, 16% for trypsin C, and 22% for Trypsin D.

The apparent caseinolytic activities at pH 4.0 were presumed to be due to a pH rise in the reaction mixture associated with the presence of the alkaline (pH 8.0) casein substrate. The caseinolytic activities of trypsin A and B declined at a pH greater than 10.0; however, trypsin C and D maintained high activity up to pH 10.5. Trypsin A had highest caseinolytic activity and trypsin B the lowest activity, with trypsin A possessing approximately 2 times higher caseinolytic activity than trypsin B and C.

The activity of trypsin D was influenced by components of the buffer solution in that enzyme activity decreased at pH

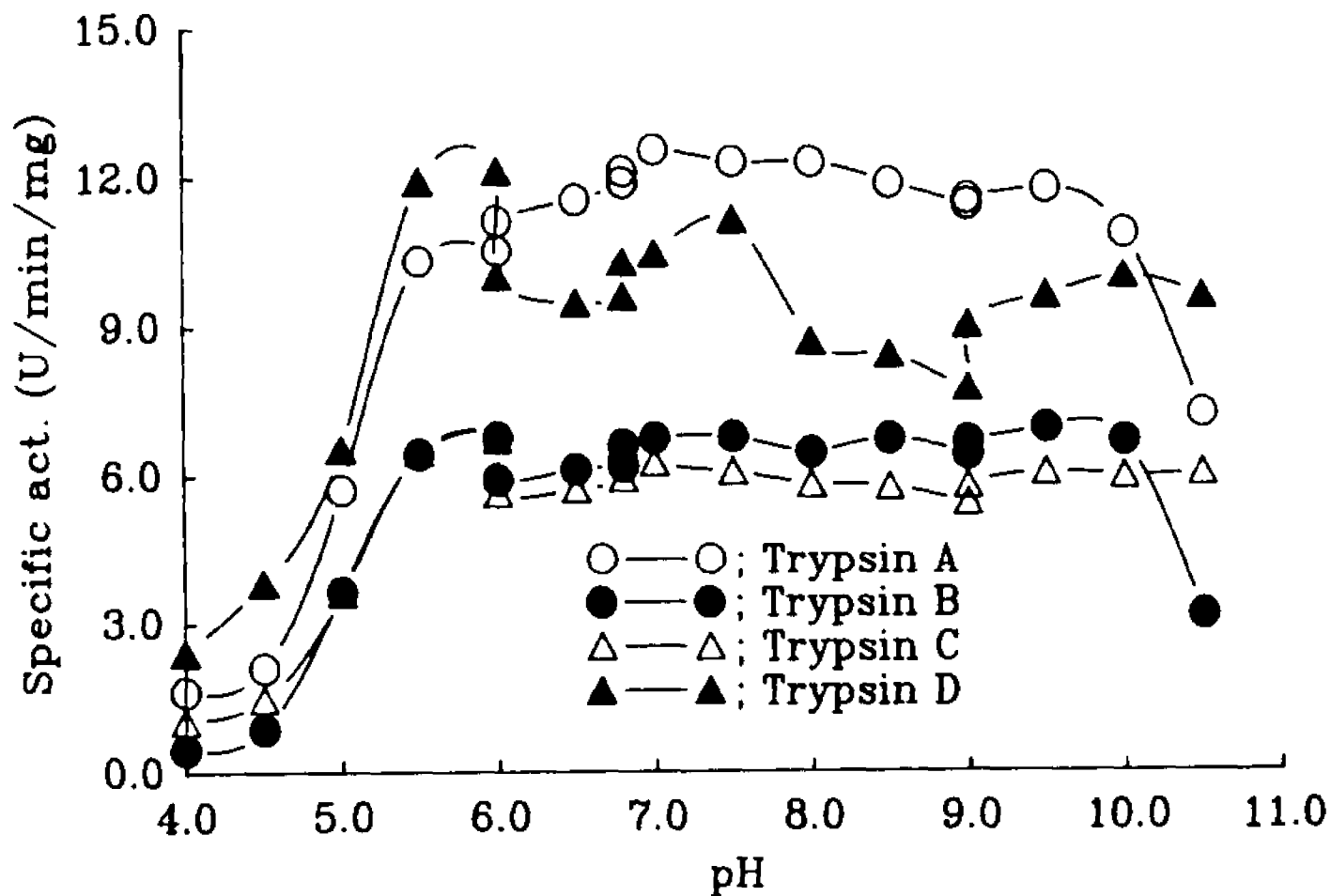


Fig. IV-12. Effect of pH on hydrolysis of casein by purified crawfish hepatopancreatic trypsins. Buffers used were 0.1 M citrate-sodium citrate (pH 4.0-6.0), 0.1 M $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ (pH 6.0-6.6), 0.1 M TES- NaTES (pH 6.6-8.0), 0.1 M Tris-HCl (pH 8.0-9.0), and 0.1 M $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$ (9.0-10.5).

8.0 to 9.0 when using Tris-HCl buffer solution. Citrate buffer had higher caseinolytic activities than did phosphate buffer solution at pH 6.0.

A comparison of the caseinolytic activities of crawfish trypsins with other purified trypsins from crustacea or fish is difficult due to different experimental condition or different activity units. However, generally the optimum pH for proteolytic activities has been reported to be in the neutral and weak alkaline range for crabs (Brun and Wojtowicz, 1976; Muramatsu and Morita, 1981; Dendinger, 1987), lobster (Galgani and Nagayama, 1987b), Antarctic krill (Seki et al., 1977), and shrimp (Galgani et al., 1984; Honjo et al., 1990). Trypsins from fish intestine generally possess higher caseinolytic activity at alkaline pH. Fish trypsins displayed the highest caseinolytic activities at pH 9.4 to 10.0 from pyloric caeca of sardine (Murakami and Noda, 1981), mackerel (Kim and Pyeun, 1986), skipjack (Pyeun et al., 1988), anchovy (Martinez et al., 1988), and Pacific salmon (Pivnenko et al., 1990).

2). Effect of pH on Esterolytic and Amidolytic Activity

Trypsin activities against N-p-tosyl-L-arginine methyl ester (TAME) were low in acidic media and increased linearly up to a pH of 6.0. However, activities then increased rapidly to an optimum at pH 7.5-8.0 (Fig. IV-13). Trypsin B and C had higher esterolytic activities in the pH range of this study

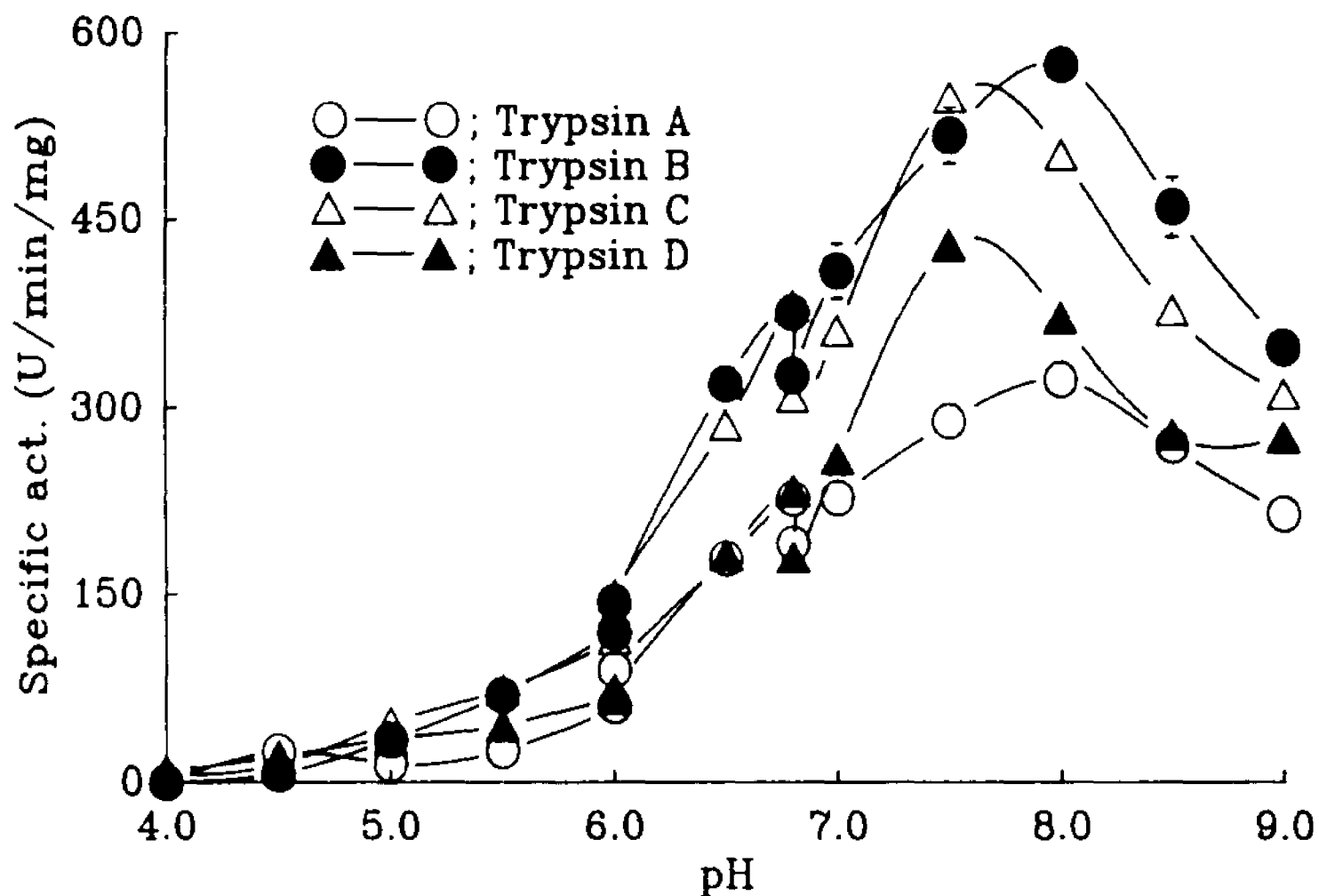


Fig. IV-13. Effect of pH on hydrolysis of N-tosyl-L-arginine methyl ester (TAME) by purified crawfish hepatopancreatic trypsins. Buffers used were the same as in Fig. IV-12 except 0.1 M TES-NaTES (pH 6.0-6.8). Reaction was performed at 25°C and indicated pH.

than trypsin A and D. The optimum pH condition for the hydrolysis of TAME at 25°C was pH 7.5 for trypsin C and D, and pH 8.0 for trypsin A and B. The specific activities of trypsin A, B, C, and D at their optimum pH for the hydrolysis of TAME were found to be 320, 570, 550, and 430 U, respectively. Contrary to caseinolytic activities, esterolytic activity of trypsin A at optimum pH, which had highest caseinolytic activity, was lowest. At physiological pH, esterolytic activities varied and their relative activities compared to maximum activities were 59% for trypsin A, 57% for trypsin B, 56% for trypsin C, and 42% for trypsin D. Lower activities in the Tris-HCl buffer solution occurred than in the same pH TES-NaTES buffer solution. Esterolytic activities of trypsin A, B, C, and D in 0.05 M TES-NaTES buffer increased 19, 15, 23, and 30%, respectively, compared with activities in 0.05 M Tris-HCl buffer.

Trypsin from Atlantic blue crab had maximal activity for TAME at pH 8.2 with no apparent activity below pH 5.0, and little activity above 9.0 (Dendinger and O'Connor, 1990). Three proteases from antarctic krill were the most active for hydrolysis of TAME at pH 8.0 (Seki et al., 1977). Trypsin-like enzymes from digestive gland of crab were maximally active at pH 7.8-8.2 for TAME (Galgani and Nagayama, 1987a), and four trypsin-like enzymes from crab had maximum activity between pH 5.3 to 7.5 (Muramatsu and Morita, 1981). Apparent pH-activity profiles of four tryptsins from crawfish hepatopancreas were

similar to pH profiles from other marine invertebrates (Osnes and Mohr, 1985b; Dendinger and O'Connor, 1990) or teleost fish (Cohen et al., 1981a; Hjelmeland and Raa, 1982; Simpson and Haard, 1984a; Asgeirsson et al., 1989).

The hydrolysis of p-benzoyl-D,L-arginine p-nitroanilide (BAPNA) was examined for each of trypsin-like enzymes (100 $\mu\text{g/ml}$) from crawfish hepatopancreas at 45°C and in the pH range of 5.5 to 9.0 (Fig. IV-14). Measurement of amidase activities was difficult over pH 9.0 or under pH 5.5, due to formation of a white precipitate of calcium carbonate at the high pH, and low activities at low pH. The optimum pH for the hydrolysis of BAPNA was found to be pH 8.0 for trypsin C, whereas amidolytic activities of the other three trypsins were not different in neutral and alkaline pH range. The order of activity was trypsin C > B > A > D in the experimental pH range. Specific activities of enzyme A, B, C, and D at optimum pH were found to be 0.6, 1.3, 2.3, and 0.31 U, respectively. Enzyme C had about 7.4-fold higher activity than did enzyme D at optimum pH. At physiological pH, relative activities for all of the trypsins were approximately 75% of their maximum activities, which establishes higher relative amidase than esterolytic activities.

Amidase activity profiles over the entire experimental pH range for trypsin A and D were very broad due to minimal effect of pH. This is similar to trypsin from shrimp hepatopancreas which had a broad pH optimum in the pH range of

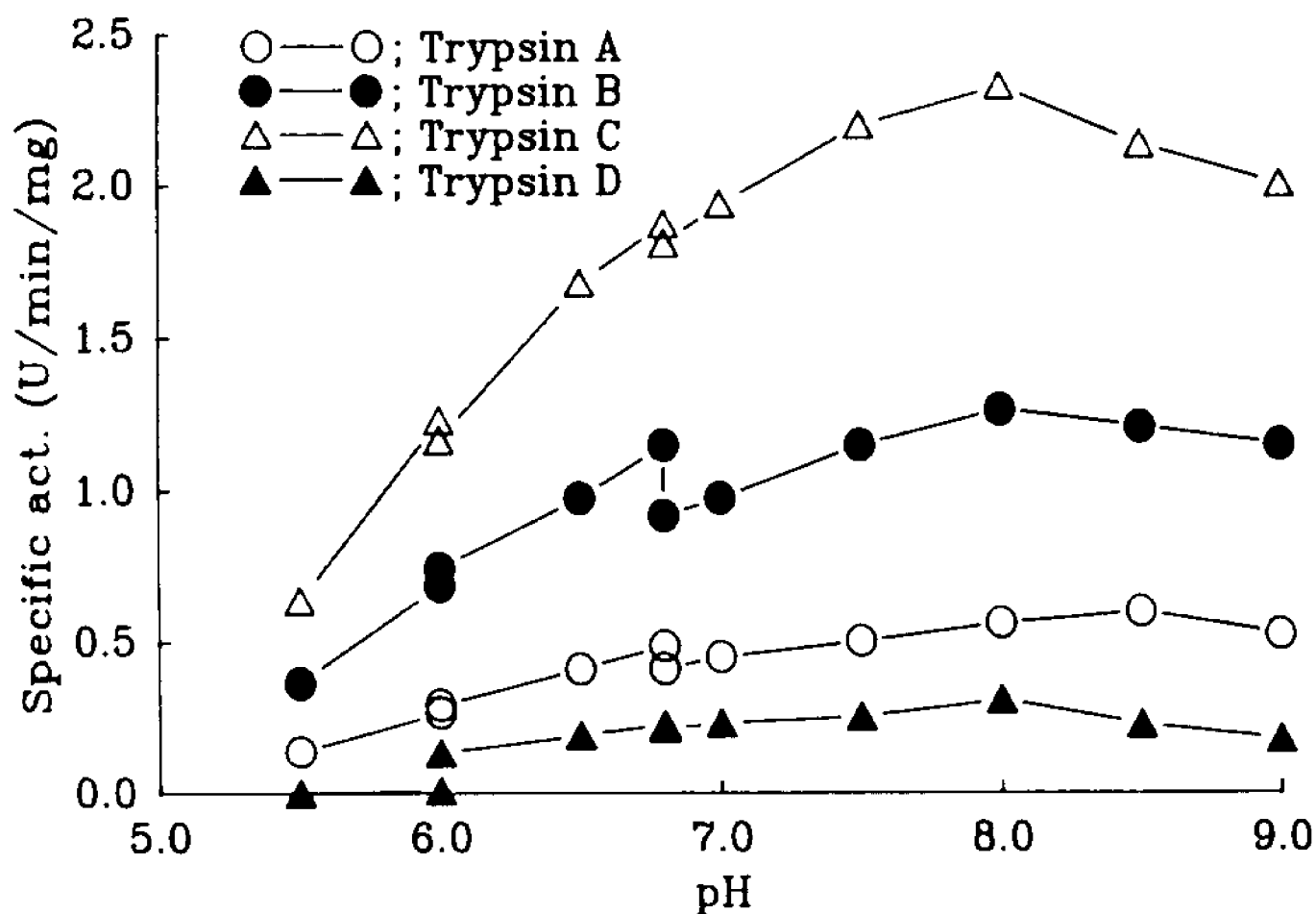


Fig. IV-14. Effect of pH on hydrolysis of benzoyl-D,L-arginine-p-nitroanilide (BAPNA) by purified crawfish hepatopancreatic trypsins. Buffers used were 0.1 M citrate-sodium citrate (pH 5.5- 6.0), 0.1 M TES-NaTES (pH 6.0-6.8), and 0.1 M Tris-HCl (pH 6.8-9.0). Reaction was performed at 45°C and indicated pH.

6.5 to 11.0 for BAPNA. The optimum pH of 8.0 for trypsin B and C is similar to that of Japanese spiny lobster trypsin-like fraction (Galgani and Nagayama, 1987b), bovine pancreatic trypsin (Erlanger et al., 1961), and capelin trypsin (Hjelmeland and Raa, 1982). Amidase activities of trypsin A and D were lower at the optimum pH than trypsin B and C, which is similar to esterolytic activity, but is opposite of caseinolytic activity.

Although trypsin B and C had similar levels of caseinolytic and esterolytic activities in the entire pH range, amidase activities were variable and specific activity of trypsin C was almost 2-fold, than trypsin B. Therefore, specific activity of enzyme is substrate dependent.

B. Effect of Temperature on Trypsin Activity

1). Effect of Temperature on Proteolytic Activity

Trypsin A and B had maximum caseinolytic activities at 45°C, whereas, trypsin C and D showed maximum activity at 50°C (Fig. IV-15). The maximum specific activities of trypsin A, B, C, and D were found to be 12, 6.5, 9.6, and 14 U, respectively at optimal temperature. The specific activities of trypsin A, B, C, and D at the ambient temperature of 25°C were similar and exhibited approximately 30% of maximal activities. Thus, these enzymes demonstrated considerable activity at physiological temperature. All of the enzymes were inactivated

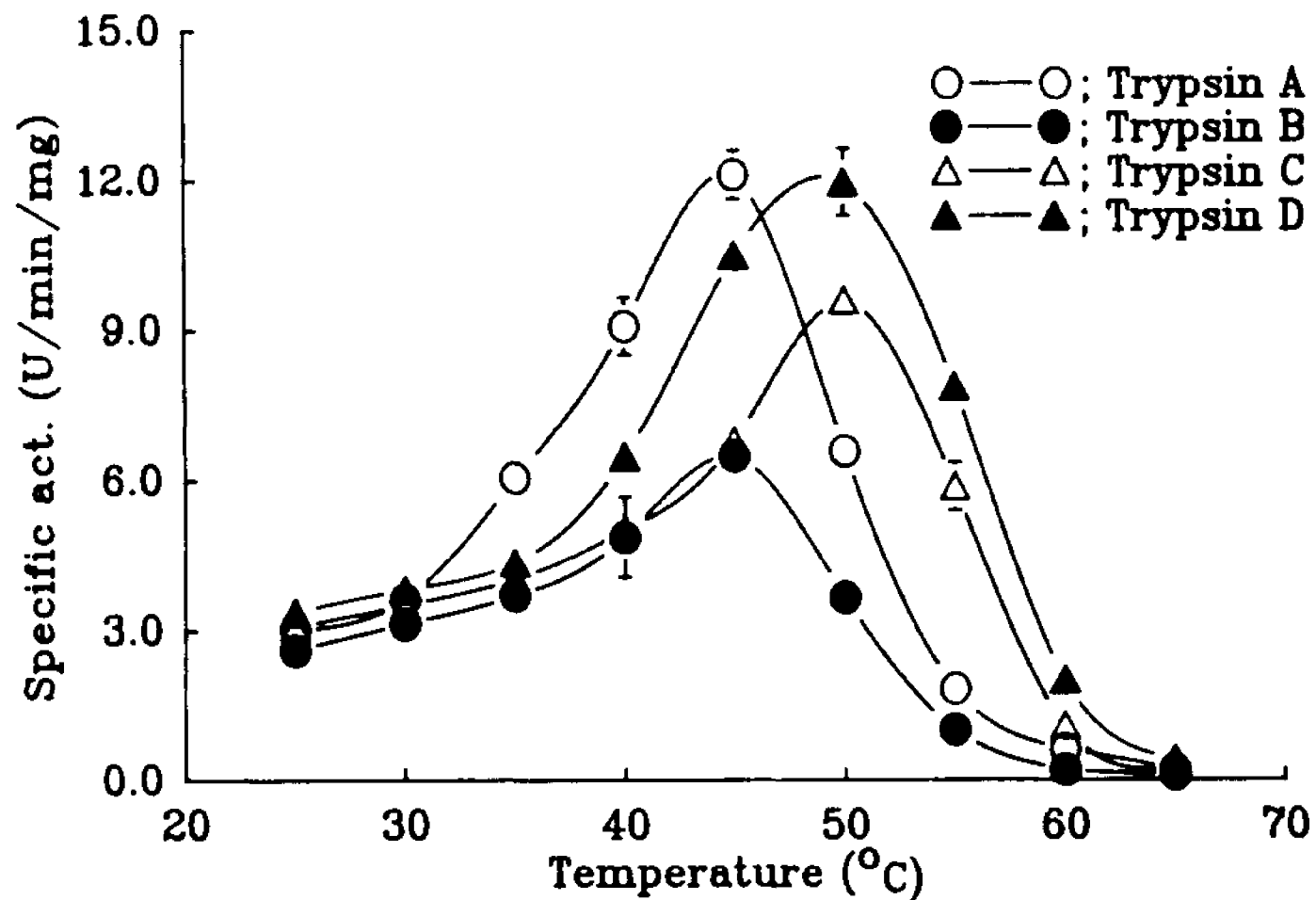


Fig. IV-15. Effect of temperature on hydrolysis of casein by purified crawfish hepatopancreatic trypsins. Reaction was performed for 20 min at pH 6.8 (0.1 M Na_2HPO_4 - NaH_2PO_4) and indicated temperature.

at 65°C due to heat denaturation.

The results show that optimum temperatures of purified trypsins are somewhat less than the temperature optima determined in the crude enzyme extract. In the latter, optimum temperature of caseinolytic activity was 50°C, which was still high at 55°C. Higher optimum temperature of crude enzyme preparations than purified enzymes is possible due to the presence of other proteins that stabilize enzyme proteins (Osnes and Mohr, 1985b). Also, optimum temperature was shifted to higher temperature in the presence of substrates or other low molecular weight effectors of enzymatic activity (Wedler and Hoffmann, 1974; Hachimori et al., 1974). Crude trypsin preparations from the digestive extract of crustacea have optimum temperature for proteolytic activities at 50°C for two species of shrimp (Galgani et al., 1984) and at 45 to 55°C for five species of crab (Galgani and Nagayama, 1987b; Galgani and Nagayama, 1988). The digestive protease from lobster has been found to have higher optimum temperature (60°C) than that from crab and shrimp (Galgani and Nagayama, 1984, 1986). Purified alkaline proteinases from intestine of teleost fish, such as sardine (Murakami and Noda, 1981), skipjack (Pyeun et al., 1988), and mackerel (Kim and Pyeun, 1986) have been reported to have an optimum temperature range similar to that of crustacea. Proteolytic enzymes active in the neutral and alkaline pH range from the digestive tract of marine gastropods had optimum temperature in the range of 45-55°C,

but an extremely low optimum temperature of 30°C was demonstrated for sea hare (Cho et al., 1983). Hara et al. (1984) reported that two alkaline proteases from rotifer, Brachionus plicatilis, showed maximum caseinolytic activity at 37°C.

2). Effect of Temperature on Esterolytic and Amidolytic Activity

Trypsins from crawfish had maximum esterolytic activities for N-p-tosyl-L-arginine methyl ester (TAME) at 60°C, but trypsin B and C retained high activities up to 65°C at pH 6.8 (Fig. IV-16a). The specific activities of trypsin A, B, C, and D were 780, 1,200, 1,200, and 900 U, respectively, at optimum temperature. The relative activities of trypsin A, B, C, and D at a temperature of 25°C were 39, 37, 29, and 35% of their maximum activities, which indicates that considerable activity is maintained even at environmental temperatures. Trypsin B and C were found to have higher thermal stability than trypsin A and D. All tryptic activities increased linearly up to their optimum temperature and all of the enzymes were inactivated at 70°C.

The temperature dependence of esterolytic activities at pH 8.1 is illustrated in Fig. IV-16b. Trypsin A and B had maximum esterolytic activities at 55°C, which is a lower optimum temperature than obtained at pH 6.8. Trypsin C and D had optimum temperature at 60°C, similar to that at pH 6.8.

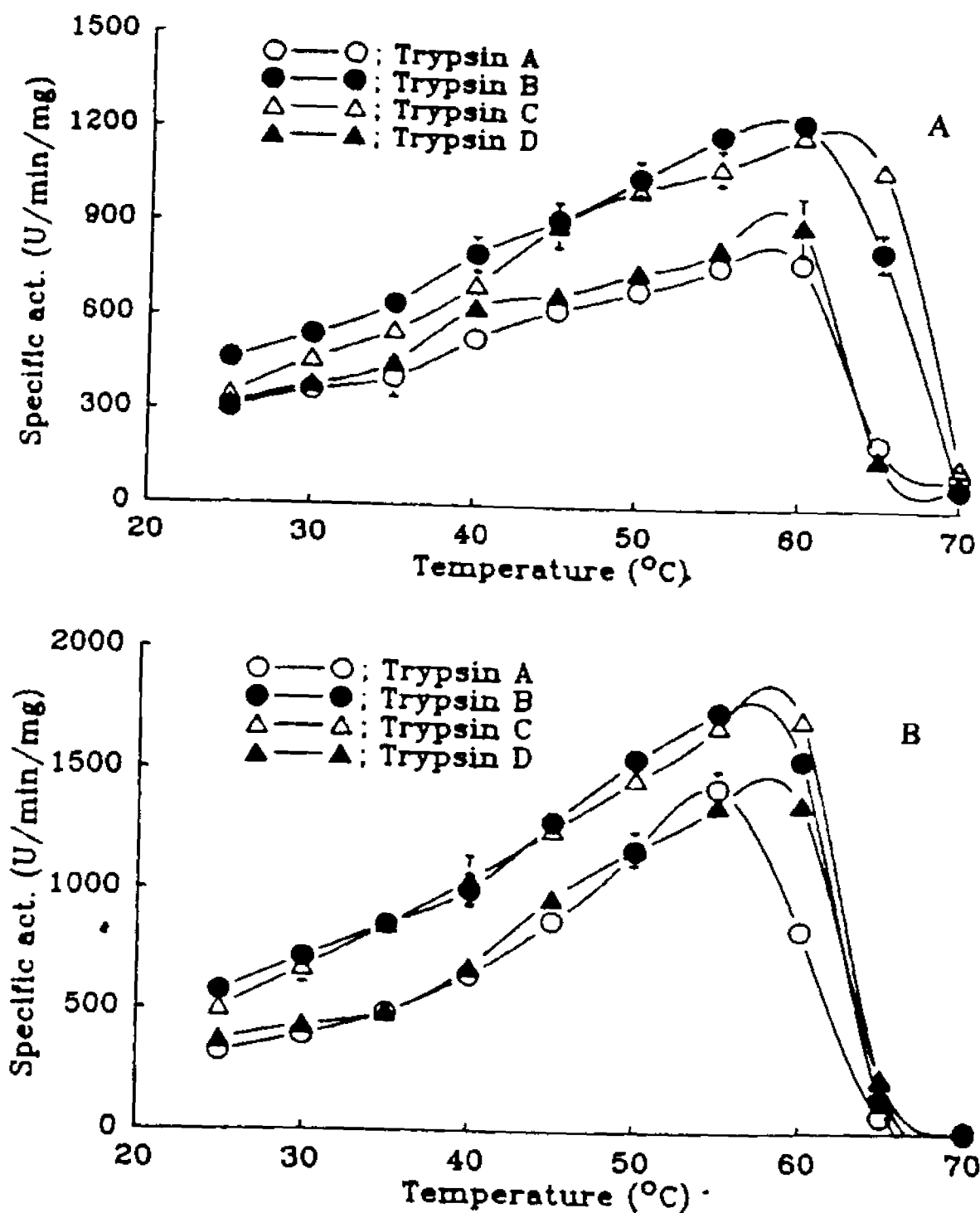


Fig. IV-16. Effect of temperature on the hydrolysis of N-tosyl-L-arginine methyl ester (TAME) at pH 6.8 (A) and pH 8.1(B) by purified crawfish hepatopancreatic trypsins.

The specific activities of trypsin A, B, C, and D at pH 8.1 were found to be 1,200, 1,700, 1,700, and 1,400 U, respectively. The relative activities of trypsin A, B, C and D at a temperature of 25°C were 28, 33, 30, and 27% of their maximum activities. These relative activities were somewhat lower than at physiological pH. Specific activities of trypsin A and D at pH 8.1 were 1.5-fold higher than that at physiological pH, and enzyme B and C were 1.4-fold higher.

Trypsin A, B, and D had maximum amidase activities for benzoyl-D.L-arginine-p-nitroanilide (BAPNA) at 60°C, whereas, trypsin C gave maximum activity at 65°C (Fig. IV-17). Specific activities for enzyme A, B, C, and D were 1.3, 2.6, 5.5, and 1.3 U, respectively, at pH 6.8 and optimum temperature. Their specific activities at 25°C were 12, 10, 5, and 1% of maximum activity. Compared with caseinolytic or esterolytic activities, specific activity at 25°C was low for all enzymes.

Trypsin A, B, and D were completely inactivated at 70°C, but trypsin C exhibited 26% of its maximum activity, which means the enzyme had the highest thermostability. Although esterolytic activities of enzyme B and C for TAME had similar temperature-activity profiles, the temperature-activity profile of amidase activities evidenced distinct differences, i.e., amidase activity of enzyme C was about two-fold higher than enzyme B. Specific activity of enzyme C was 4.4-fold higher in this study than enzyme D, which is a notably higher ratio than that of caseinolytic or esterolytic activity.

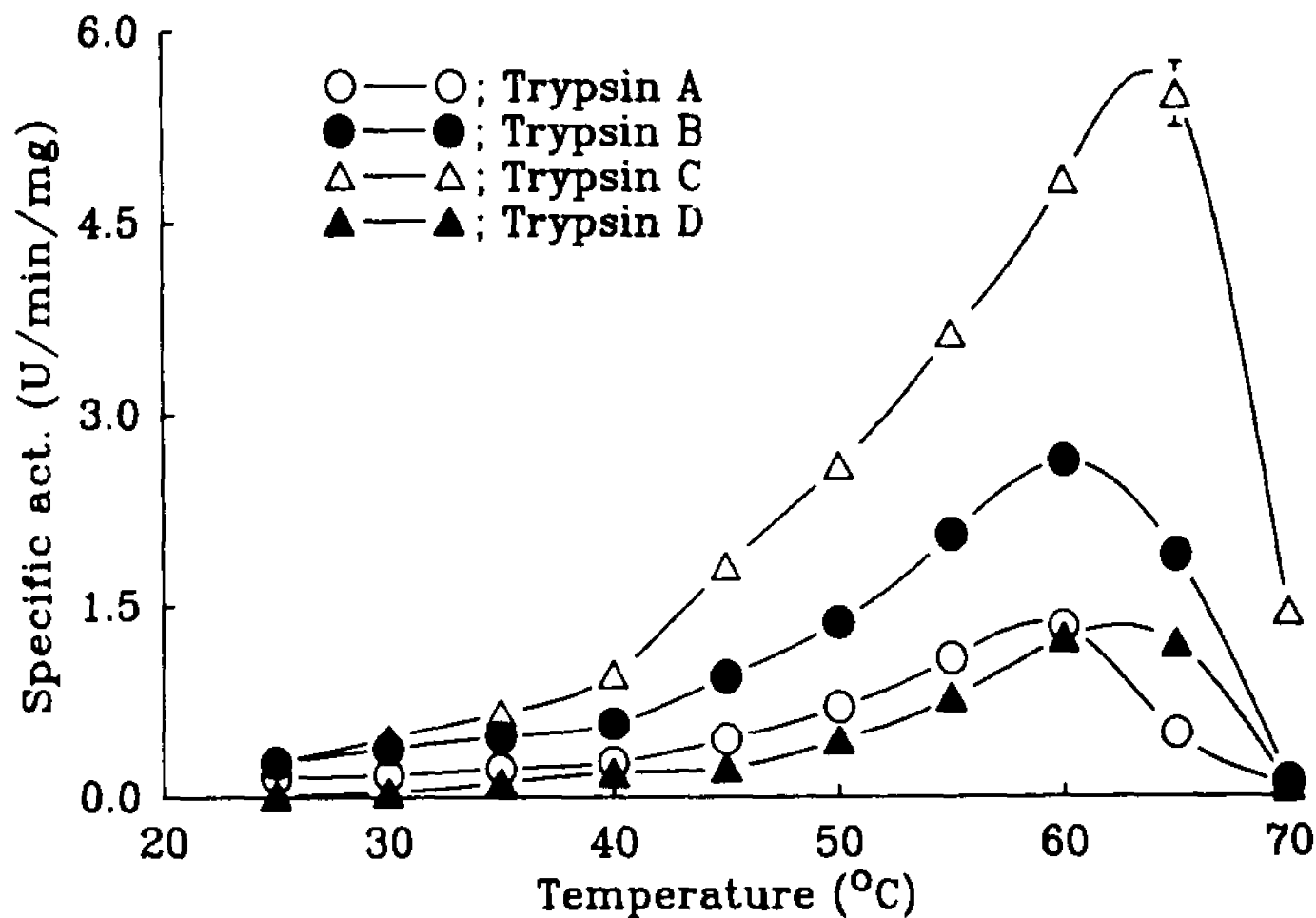


Fig. IV-17. Effect of temperature on hydrolysis of benzoyl-D,L-arginine-p-nitroanilide (BAPNA) by purified crawfish hepatopancreatic trypsins. Reaction was performed at pH 8.1 and indicated temperature.

Enzyme A and D had lower activities than enzyme B and C for amidase and esterase activities, but enzyme A had highest caseinolytic activity. Therefore, temperature optima of crawfish trypsins are dependent on both pH and type of substrate.

Optimum temperature of enzyme activity is dependent on reaction time, which has been demonstrated by Osnes and Mohr (1985b, 1986). They showed that the optimum temperature for three trypsin-like enzymes from Antarctic krill shifted to low temperature with longer reaction time. Trypsins from Arctic capelin (Hjelmeland and Raa, 1982) and Greenland cod (Simpson and Haard, 1984a) had maximum activities for TAME at 42°C and 30-40°C, respectively. Although the environmental temperature of Antarctic krill is low, the maximum esterolytic activities of trypsins from krill was in the range of 50 to 54°C (Osnes and Mohr, 1985b), which is higher than Arctic fish species. However, optimum temperatures of trypsin from fish were reported as 50-55°C for Atlantic cod (Asgeirsson et al., 1989; Shin and Zall, 1986) and 55-60°C for milkfish (Chen et al., 1989). Also, activity of trypsin-like enzymes from the digestive extract of crustacea had optimum temperature at 50°C for two species of shrimp (Galgani et al., 1984), 45°C for shrimp (Honjo et al., 1990), and around 50°C for five species of crab (Galgani and Nagayama, 1987a). Higher optimum temperatures were reported for trypsin from the digestive tract of lobster (60°C) (Galgani and Nagayama, 1987b) and

Atlantic blue crab (70°C) (Dendinger and O'Connor, 1990).

C. Determination of Thermal Stability

The residual esterolytic activities of purified trypsins from crawfish hepatopancreas were examined with 1 mM TAME at 25°C and at pH 6.8 after 5 min of heat treatment at various temperatures (Fig. IV-18). Heat treatment of purified enzymes at 45°C for 5 min caused a slight reduction in tryptic activity. Heat treatment at 55°C resulted in approximately 70% reduction of activity for enzyme A, B, and D, and 60% reduction for trypsin C. This result was somewhat different from the result from temperature-activity profile (Fig. IV-16A). This indicates that thermal stability of enzyme is enhanced in the presence of substrate (Wedler and Hoffmann, 1974). All enzymes were nearly completely inactivated at 60°C. Although thermostabilities among the four trypsins were not distinctively different, enzyme C had slightly higher thermal stability than the other enzymes. This observation coincides with results from the effect of temperature on enzyme activity, in which enzyme C also appeared to possess greater thermostability.

Thermal stability of protein was also studied with casein solution (5%) added to the purified enzyme solutions. Thermal stability increased considerably in the presence of substrate or other low molecular weight effectors of enzyme activity (Parfait, 19743; Wedler and Hoffmann, 1974; Hachimori et al.,

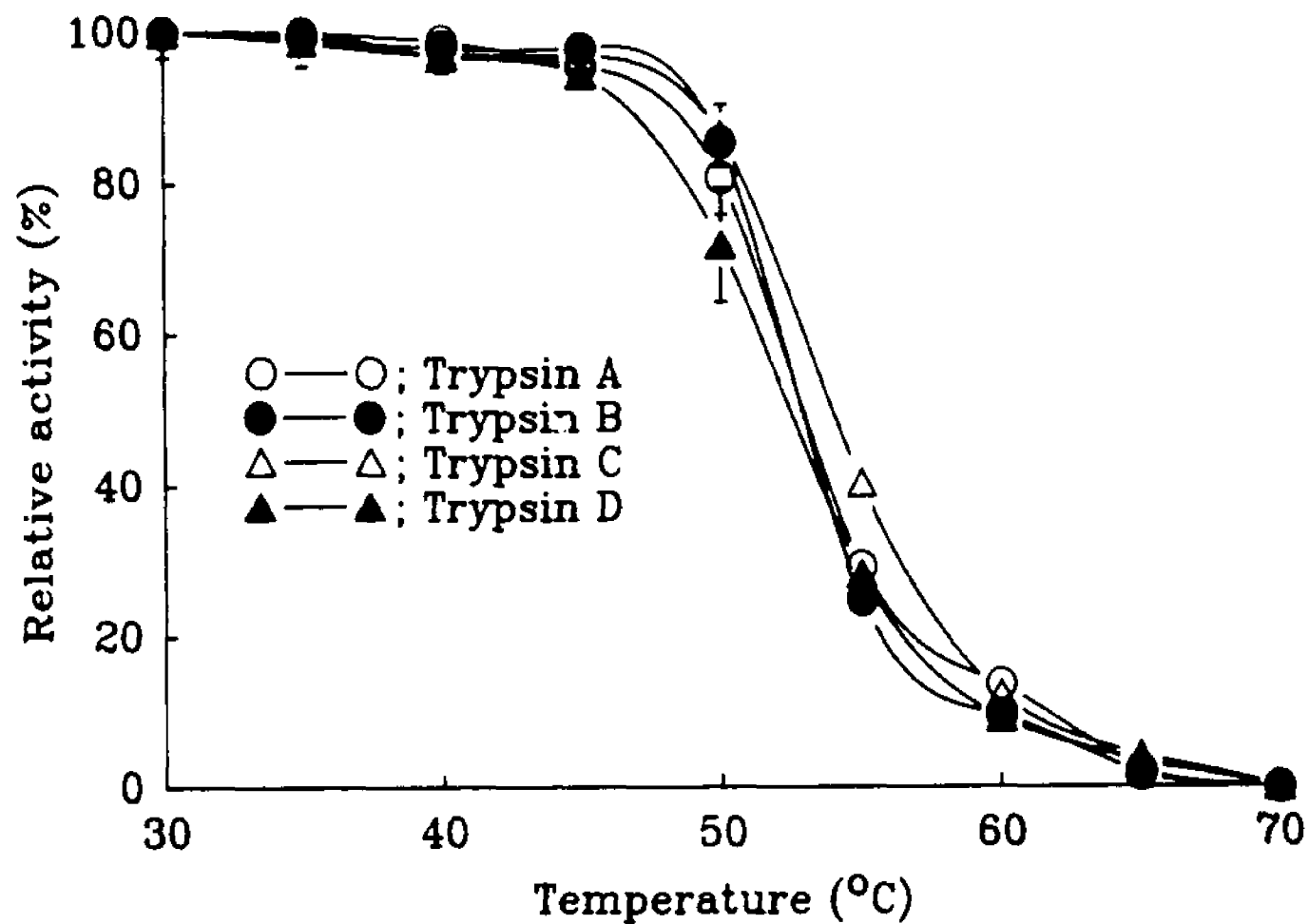


Fig. IV-18. Thermal stability of purified crawfish hepatopancreatic trypsins. Trypsins were incubated for 5 min at indicated temperature and residual activities were determined with 1 mM TAME at pH 6.8 and 25°C.

1974). As indicated in Fig. IV-19a and 19b, thermal stabilities of all enzymes increased in the presence of casein. Heat treatment of purified enzymes in the absence of casein at 40°C for 20 min caused a slight activity reduction in trypsin A. Other trypsins were not inactivated by heat treatment at 40°C. Incubation at 45°C induced a slight activity reduction for all trypsins incubated without casein, whereas incubation with casein evidenced no change in activities. Heat treatment at 50°C without casein induced approximately 70, 60, 50, and 20% activity reduction in trypsin A, B, C, and D, respectively. When incubated in the presence of casein, a lower reduction of enzyme activity was observed, especially with enzymes C and D.

These results suggest that the surrounding environment influences enzyme stability. Partially purified enzymes were more stable against thermal inactivation than purified enzymes (Osnes and Mohr, 1985b). Hence, it can be assumed that the presence of protein enhanced thermal stability of enzymes.

Trypsin from Atlantic blue crab was stable from 30 to 50°C for 30 min preincubation but activity was rapidly lost above 50°C (Dendinger and O'Connor, 1990). Tryptic activities from the hepatopancreas of jonah and rock crab were reduced 60% of original activities by incubation at 50°C for 20 min but the enzymes were stable at 40°C for 20 min (Brun and Wojtowicz, 1976). These findings are in good agreement with present result. Three alkaline proteinases from pyloric caeca

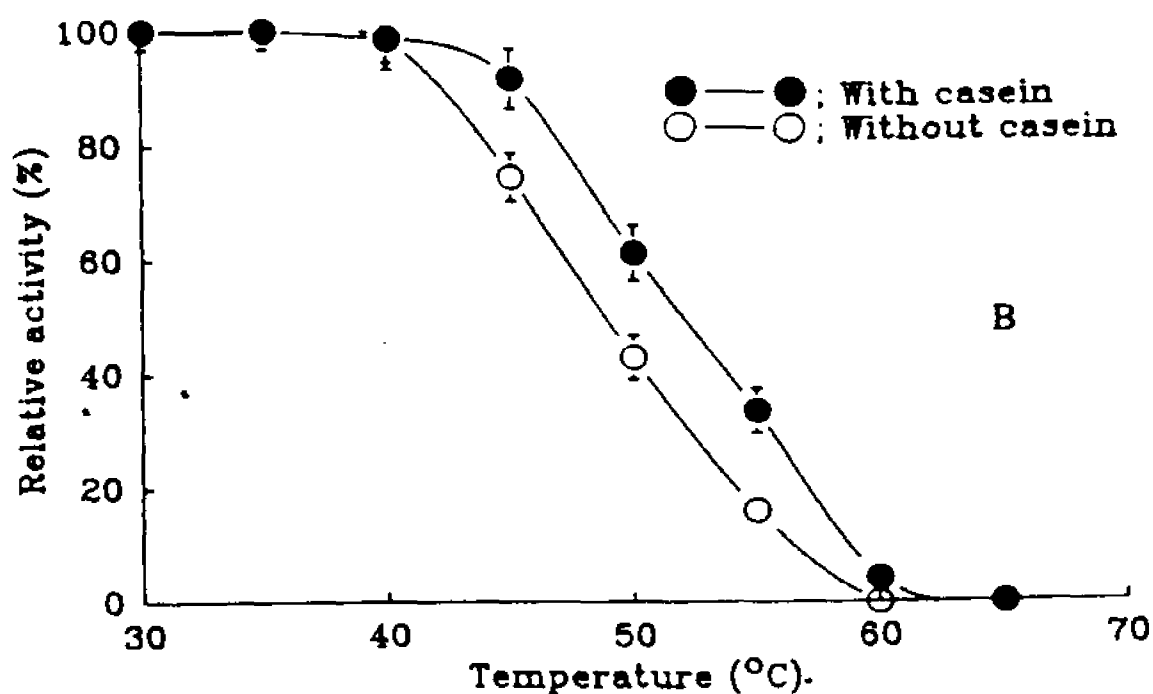
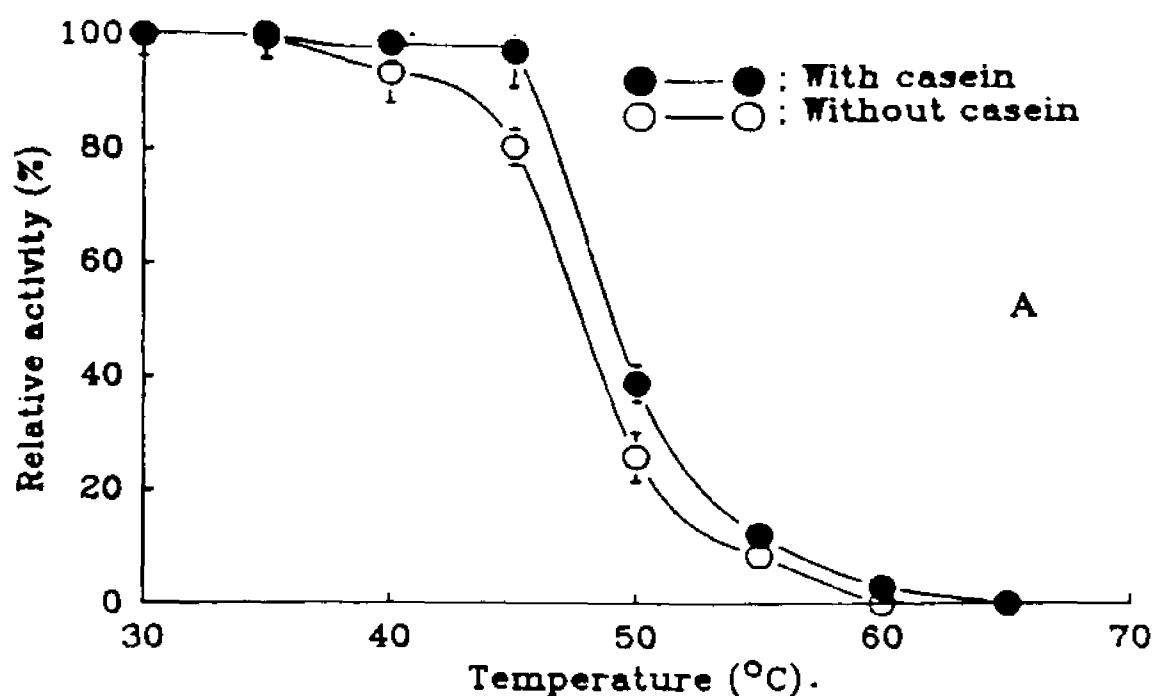


Fig. IV-19a. Thermal stability of purified crawfish hepatopancreatic trypsin A and B in the presence or absence of casein. Trypsins were incubated for 15 min at indicated temperature and residual activities were determined with 5% casein at pH 6.8 and 45°C.

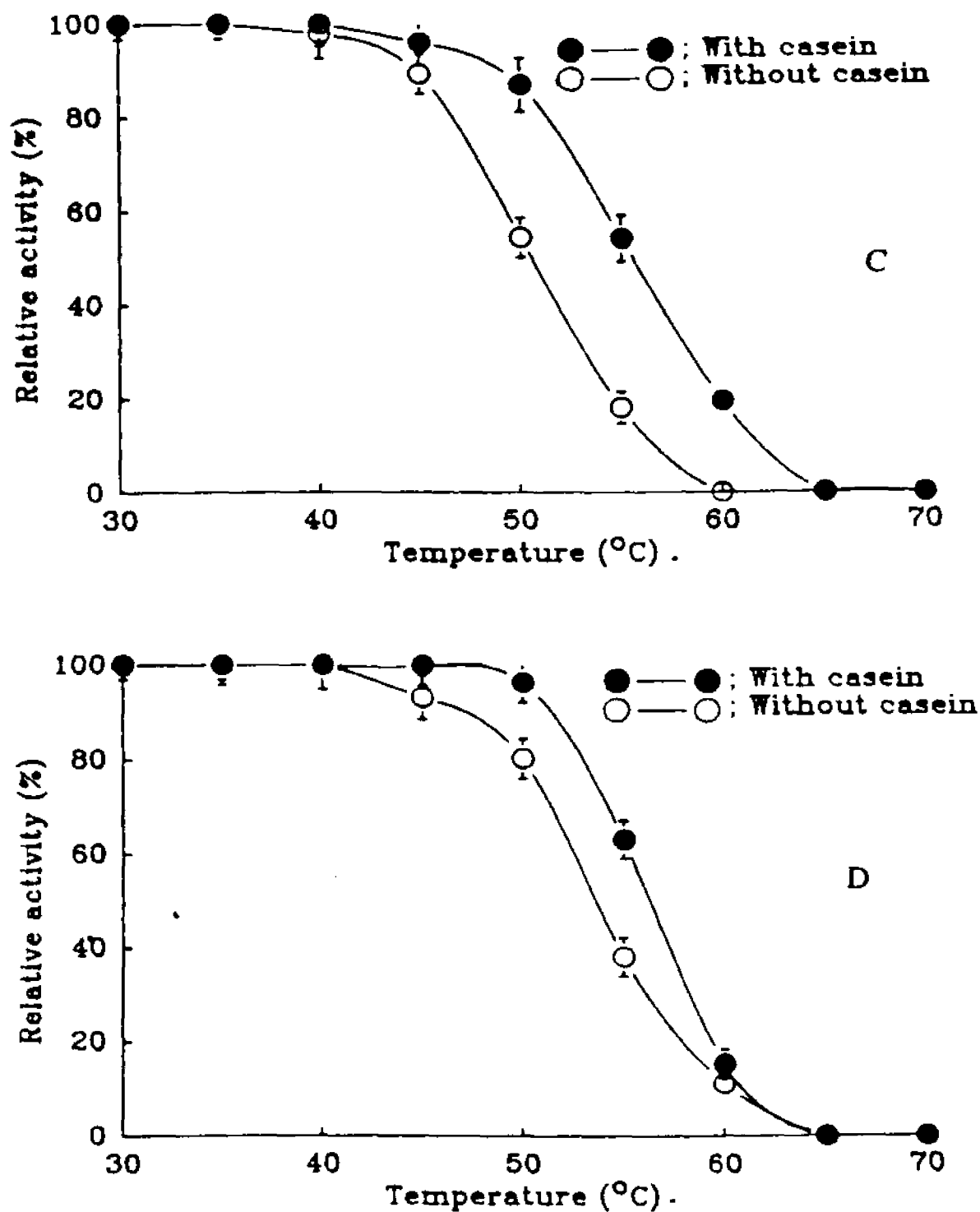


Fig. IV-19b. Thermal stability of purified crawfish hepatopancreatic trypsin C and D in the presence or absence of casein. Reaction conditions were the same as in Fig. IV-19a.

of mackerel (Kim and Pyeun, 1986) were stable when heated for 5 min at 45°C but most activity was lost at 50°C. Uchida et al. (1984) reported that two anionic trypsins were stable at 35°C, however, activities decreased with increased heating temperature, and were inactivated at 55°C.

More numerous disulfide linkages as well as stronger hydrophobic interactions in the interior of protein contribute to greater thermostability of proteins (Komatsu and Feeney, 1970; Sundaran et al., 1980). Disulfide bonds may stabilize a folded conformation that is no longer thermodynamically stable (Creighton, 1983). A purified cod trypsin had higher activity and a lower activation energy than bovine enzyme although the fish enzyme was less stable than the bovine at temperatures above 40°C (Simpson and Haard, 1984a). These authors suggested that this might be caused by the lower number of maximal intramolecular disulfide bonds in fish trypsin (4) compared to bovine trypsin (6).

D. Determination of pH Stability

Enzyme stability in the physiological pH range is important to the storage stability of enzyme. Each trypsin solution (20 µg/ml) was mixed with the same volume of buffer solution i.e., 0.1 M glycine-HCl (pH 2.0), 0.1 M citrate-sodium citrate (pH 4.0 and 5.6), 0.1 M Tris-HCl (pH 6.8 and 8.1) or 0.1 M glycine-NaOH buffer (pH 10.0) and incubated (Fig. IV-20a and 21b). Trypsins were stable at pH 6.8 and 8.1

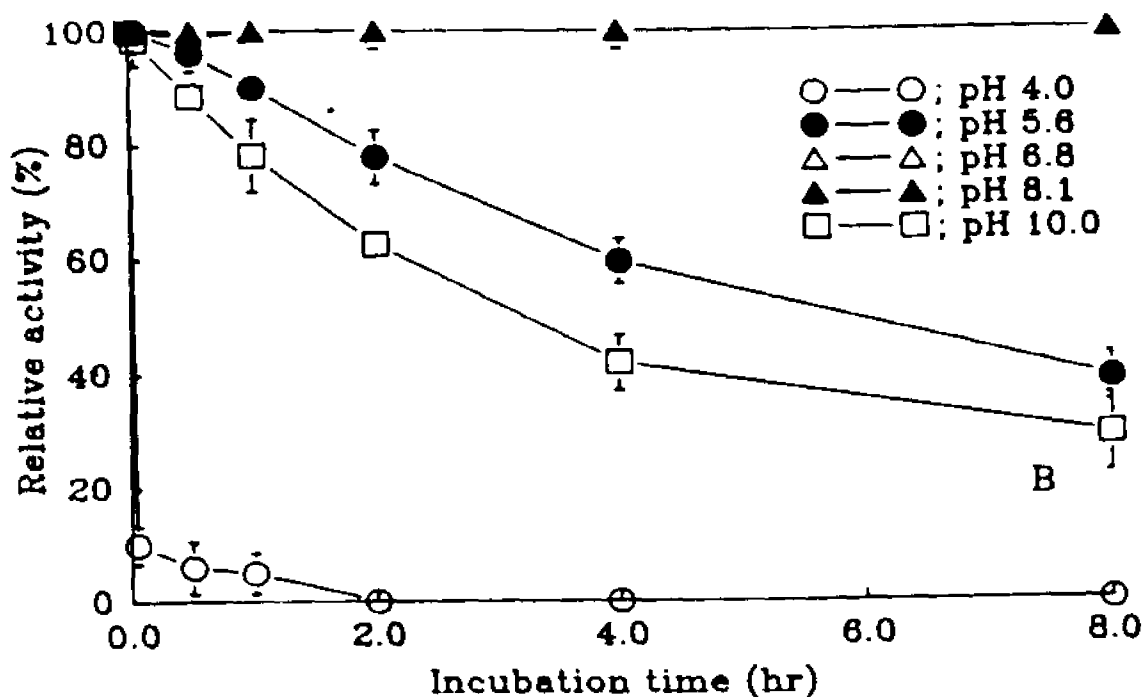
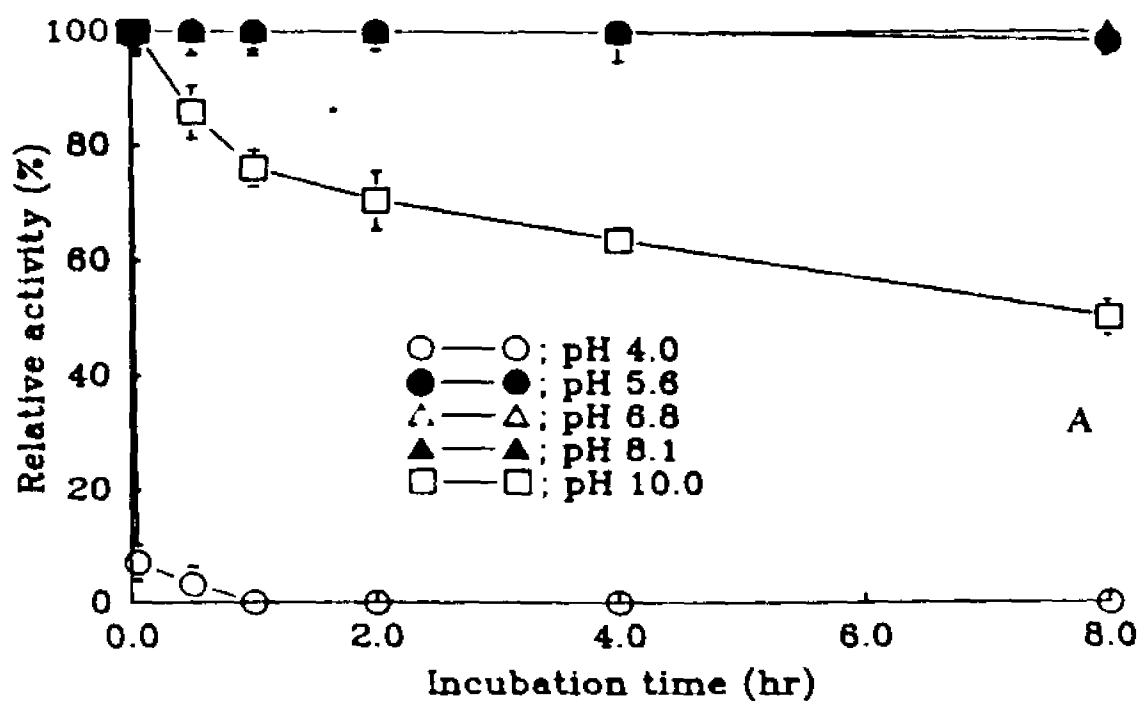


Fig. IV-20a. pH-stability of purified crawfish hepatopancreatic trypsin A and B. Reaction conditions were described in materials and methods section.

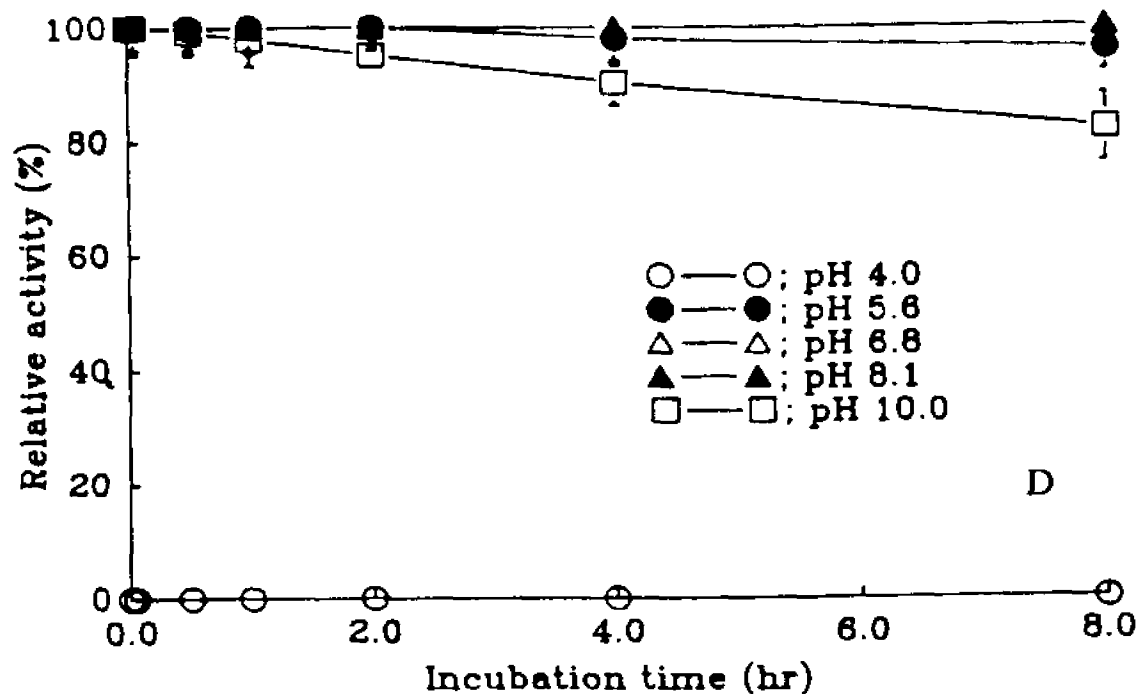
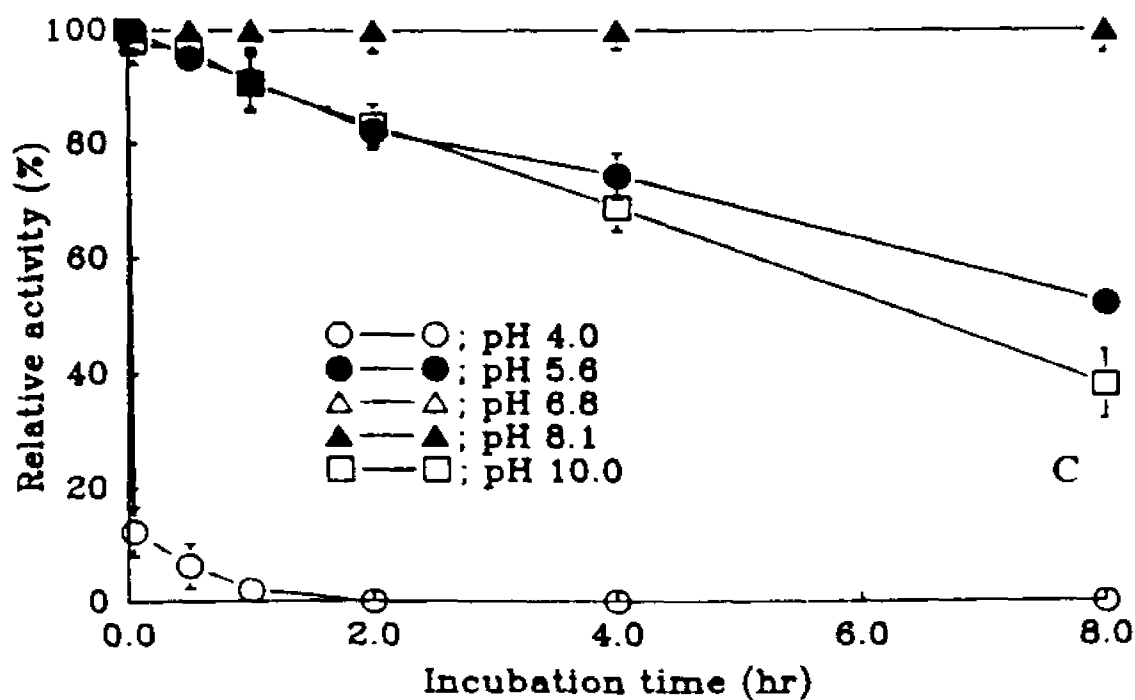


Fig. IV-20b. pH-stability of purified crawfish hepatopancreatic trypsin C and D. Reaction conditions were the same as in Fig. IV-20a.

for up to 8 hr incubation. At pH 5.6, activities of trypsin A and D did not change during 8 hrs, but activity of trypsin B and C decreased to 45 and 55%, respectively, of their original activity. At pH 10.0, activities of trypsin A, B, C and D decreased to 50, 30, 40, and 80% of their original activities after 8 hr incubation. Trypsin D was most stable at neutral and alkaline pH, and trypsin B was rapidly denatured at pH 5.6 and 10.0 after 4 hr incubation. However, after incubation of trypsin at pH 2.0 (data not shown) and 4.0, activities were rapidly lost due to irreversible denaturation. This was confirmed when trypsins were dialyzed overnight against 0.05 M Tris-HCl buffer solution (pH 8.1) after treatment with pH 2.0 and 4.0 buffer solution and tryptic activities were not recovered.

Three trypsins from krill Euphausia superba were inactivated in less than 1 hr at pH 4.5, whereas they could be stored at pH 7.5 for 17 days without significant loss in activity (Osnes and Mohr, 1985b). Trypsin from krill was found to be quite stable in weak alkaline media. The enzyme decreased slowly at pH 9.6 (Osnes and Mohr, 1985b) as occurred for trypsin from shrimp (Gates and Travis, 1969). Bovine pancreatic trypsin, on the other hand, is very stable in acidic pH. Apparently, inactivation at acid pH is a phenomenon common to anionic trypsins from various species, such as crayfish (Zwilling et al., 1969), shrimp (Gates and Travis, 1969), starfish (Winter and Neurath, 1970; Kozlovskaya and

Elyakova, 1974), crab (Muramatsu and Morita, 1981), capelin (Hjelmeland and Raa, 1982), Arctic krill (Kimoto et al., 1983), Greenland cod (Simpson and Haard, 1984a), spiny lobster (Galgani and Nagayama, 1987b), and Atlantic blue crab (Dendinger and O'Connor, 1990).

Lysine and arginine are very low while acidic amino acids are very high in the crawfish enzymes compared with bovine trypsin (Table IV-2). The greater stability of the crawfish trypsins can be attributed to fewer trypsin-labile bonds susceptible to autolysis. This may be true if crawfish trypsins have specificity for peptide bonds involving the carboxyl group of lysine or arginine residues (Osnes and Mohr, 1985b). Also, the high content of acidic amino acids in the enzyme molecule may be distributed on the surface of molecules, which would cause enzyme molecules to be repelled from each other. This repulsion could be the basis for stability against autolysis.

E. Effect of Calcium Ions on the Enzymatic Activity

The effect of Ca^{2+} ion concentration on tryptic activity was determined at 25°C with 1 mM TAME. Trypsin A, B, C, and D had maximum activity at 0.5, 0.05, 0.025, and 0.1 mM, respectively, of Ca^{2+} ion concentration (Fig. IV-21). The activity of trypsin A increased to 130% at 0.5 mM Ca^{2+} ion concentration. Trypsin B and C were not influenced by addition of 0.01 mM Ca^{2+} ion, and activation was achieved at Ca^{2+}

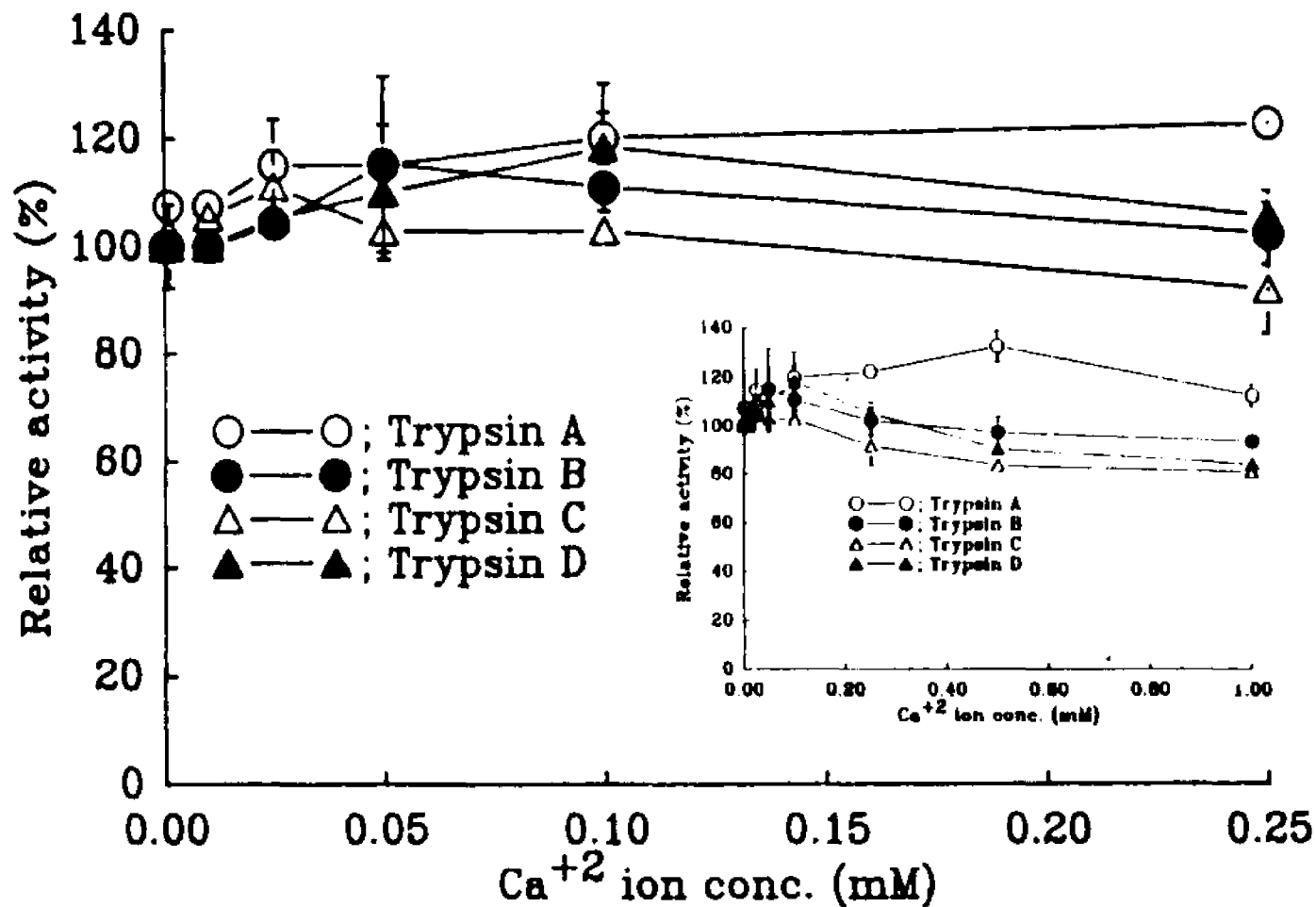


Fig. IV-21. Effect of Ca^{2+} ion concentration of the esterolytic activity for N-tosyl-L-arginine methyl ester (TAME) by purified crawfish hepatopancreatic trypsins. Reaction conditions were described in materials and methods section.

concentrations of 0.05 and 0.025 mM, respectively. Trypsin D had maximum esterolytic activity at 0.1 mM Ca^{2+} ion concentration and activity decreased to about 80% with concentrations over 1 mM Ca^{2+} ion. The activity of trypsin A was higher than the control up to a 1 mM Ca^{2+} ion concentration, but tryptic activities of the other trypsins decreased above 1 mM Ca^{2+} ion concentration.

These results are in partial agreement with previous findings on proteolytic activity of crude extract from crawfish hepatopancreas (Fig. IV-4) and those reported for digestive proteinases from crab (Galgani and Nagayama, 1988). However, calcium ions did not affect the tryptic activity of shrimp (Gates and Travis, 1969; Galgani et al., 1984; Honjo et al., 1990) and lobster (Galgani and Nagayama, 1987b). Moreover, tryptic activities from Atlantic blue crab (Dendinger and O'Connor, 1990) and crayfish (Zwilling et al., 1969) were inhibited by the addition of Ca^{2+} ion. Krill trypsins were stabilized against thermal inactivation by Ca^{2+} ions and optimum temperature increased with the addition of calcium ions (Osnes and Mohr, 1987b). Bovine pancreatic trypsin underwent rapid autolysis in the presence of calcium, particularly in alkaline solutions, and the enzyme required Ca^{2+} ions for activation. However, crawfish trypsins were very resistant to autolysis, even in the absence of CaCl_2 (Zwilling and Neurath, 1981).

F. Effect of Chemical Reagents on Enzyme Activities

Activity of all four trypsins from crawfish hepatopancreas were inhibited by soybean trypsin inhibitor (SBTI), phenylmethylsulfonylfluoride (PMSF), and diisopropyl fluorophosphate (DFP) which are inhibitors of serine enzyme (Table IV-3). All four trypsins were partially inhibited (30-35%) by 1.0 μ M of SBTI, and trypsin A, C, and D were completely inhibited by 5 μ M of SBTI. Trypsin B was inhibited to a lesser degree by SBTI or DFP compared with the other enzymes. PMSF produced approximately 73-75% inhibition. SBTI and DFP have been described as strong inhibitors of trypsin from crustacea and fish intestine such as Atlantic blue crab (Dendinger and O'Connor, 1990), shrimp (Honjo et al., 1990), Antarctic krill (Osnes and Mohr, 1985b), catfish (Yoshinaka et al., 1984), and menhaden (Pyeun et al., 1990). However, the reported inhibitory effect of PMSF has been variable, with 16 and 44% for menhaden trypsin A and B (Pyeun et al., 1990), 56% for catfish trypsin (Yoshinaka et al., 1984), 65% for cod trypsin (Simpson and Haard, 1984a; Simpson et al., 1989), 80% for anchovy trypsins (Martinez et al., 1988), and 90% for capelin trypsins (Hjelmeland and Raa, 1982). Differences in inhibitory effect by PMSF may be due to instability of PMSF in solution.

N-p-Tosyl-L-lysine chloromethyl ketone (TLCK) and benzamidine are well known trypsin specific inhibitors. Trypsin C and D were completely inactivated by TLCK, but

Table IV-3. Effect of inhibitors on the esterolytic activity of trypsin-like from crawfish hepatopancreas

Inhibitor	Conc.	% Inhibition			
		A	B	C	D
TLCK	1.0 mM	92	94	100	100
Benzamidine	1.0 mM	51	64	53	51
	5.0 mM	98	99	98	97
SBTI	1.0 μ M	31	29	34	31
	5.0 μ M	97	88	99	96
PMSF	1.0 mM	75	73	74	73
DFP	1.0 mM	87	62	99	93
Leupeptin	0.04 mM	98	97	98	92
Antipain	0.03 mM	98	99	99	97
p-CMB	1.0 mM	11	4	22	0
Iodoacetate	1.0 mM	0	11	12	0
EDTA	0.1 mM	75	83	60	65
o-Phenonthroline	1.0 mM	50	50	43	50
TPCK	1.0 mM	0	0	0	0
Pepstatin	0.26 μ M	0	0	0	0
Mercaptoethanol	1.0 mM	0	0	0	0
Dithiothreitol	1.0 mM	50	48	48	49
Glutathione	1.0 mM	93	96	98	98

Each enzyme solution (0.1 ml) was (20 μ g/ml) incubated with same volume of inhibitor at 25°C for 15 min and residual activity was determined with 1 mM TAME at 25°C and pH 8.1.

TLCK; N-p-Tosyl-L-lysine chloromethyl ketone

TPCK; N-p-Tosyl-L-phenylalanine chloromethyl ketone

SBTI; Soybean trypsin inhibitor

PMSF; Phenylmethylsulfonylfluoride

EDTA; Ethylenediamine tetraacetate

p-CMB; p-Chloromercuribenzoate

DFP; Diisopropyl fluorophosphate

enzyme A and B retained some activity. TLCK inactivates only trypsin-like enzyme whose specificity corresponds to the reagent structure, i.e., alkylation of the active-center histidine (Shaw et al., 1965; Shaw, 1980). The enzyme is known to form a covalent bond with histidine in the catalytic portion and to block the substrate-binding portion of the active center of the molecule (Severin and Tomasek, 1965). Inhibition by TLCK is difficult to interpret because of possible side reactions with -SH groups; however these results are in agreement with other reports (Zwilling and Neurath, 1981; Hjelmeland and Raa, 1982; Osnes and Mohr, 1985b; Galgani and Nagayama, 1987b; Martinez et al., 1988; Dendinger and O'Connor, 1990; Honjo et al., 1990; Pyeun et al., 1990).

Tryptic activities were reduced 51 to 63% by 1 mM benzamidine, with further inhibition by 5 mM benzamidine. Also, leupeptin and antipain, which have the argininal residue at their terminal carbon that inhibits trypsin, and papain which cleaves the carboxyl side of basic amino acids, are able to inactivate the proteolytic and esterolytic activities of trypsin. These inhibitors reduced esterolytic activities almost completely for all trypsins in this study.

Trypsins from krill (Osnes and Mohr, 1985b), cod (Simpson et al., 1989) and menhaden (Pyeun et al., 1990) were inhibited nearly completely by benzamidine, but trypsin from shrimp (Honjo et al., 1990) was not inactivated when a higher concentration of benzamidine was applied. Thus, inhibition of

trypsin by benzamidine may depend on source of the enzyme. Also, leupeptin and antipain have been reported to be very efficient inhibitors for trypsins from menhaden (Pyeun et al., 1990), catfish trypsin (Yoshinaka et al., 1984), and capelin (Hjelmeland and Raa, 1982).

Ethylenediaminetetraacetate disodium (2Na-EDTA) and o-phenanthroline, which chelate the metal ions of enzymes, reduced the esterase activities of all trypsins, although, the degree of inhibition varied. Trypsin B was greatly inhibited by EDTA compared with the other trypsins. These results indicate that these enzymes require metal ions as a cofactor for activity. Furthermore, all four trypsins evidenced approximately 50% inhibition by o-phenanthroline, which is a chelator for the removal of Zn^{2+} , Mn^{2+} , or Fe^{2+} ions. This supports results obtained with EDTA.

Inhibition of fish trypsin by EDTA varies with the source of trypsin, i.e., various crustacean trypsins were inhibited approximately 20 to 35%, including shrimp (Galgani et al., 1984; Galgani and Nagayama, 1987a), and lobster (Galgani and Nagayama, 1987b). Two trypsins from menhaden viscera were inactivated almost completely by EDTA (Pyeun et al., 1990) and two anchovy trypsins evidenced 34 and 60% inactivation (Martinez et al., 1988). However, Winter and Neurath (1970) reported that EDTA only slightly inactivated a trypsin from starfish.

p-Chloromercuribenzoate (p-CMB) and iodoacetate, which

are irreversible inhibitors of sulfhydryl protease by reacting with -SH, -NH₂ and -OH groups in protein molecules, did not inactivate the trypsins to a great extent, although trypsin C was inhibited by both chemicals at higher levels compared with other trypsins. Trypsin from shrimp was not affected by iodoacetate (Galgani et al., 1984). Catfish trypsin had low inhibition by both compounds (Yoshinaka et al., 1984).

None of the enzymes isolated in this study were inhibited by N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), which is a specific inhibitor of chymotrypsin, or pepstatin (0.26 μ M), which is a specific inhibitor of acid proteases. This suggests that the four enzymes are serine type enzymes, classified as trypsin-like enzymes and lack chymotrypsin and pepsin activity.

Disulfide bonds are stable and important to stabilizing protein conformation by increasing protein hydrophobicity. Therefore, addition of reducing agents will break disulfide bonds and cause inactivation. Dithiothreitol (DTT) reduced trypsin activities about 50%, and glutathione also showed over 90% inactivation. However, mercaptoethanol did not affect tryptic activities. Thus data obtained from reducing agents indicates the possibility that the cysteines existed as disulfide linkages in the protein molecules. Wolz and co-worker (1990) reported that sulfhydryl reagents had strong inhibitory effects on Astacus protease in the order of dithiothreitol, 2-mercaptoethanol, and glutathione. Also, two

trypsins from anchovy intestine were inhibited 15 and 18% by 2.5 mM of mercaptoethanol (Martinez et al., 1988), however, trypsins from five species of shrimp were not affected by 2 mM of mercaptoethanol (Galgani and Nagayama, 1987a).

The Present enzymes were shown to be true members of the trypsin family of serine proteases, similar to trypsin-like enzymes in crustacean (Dendinger and O'Connor, 1990; Dendinger, 1987; Galgani and Nagayama, 1987; Zwilling and Neurath, 1981). Confirmation included determination of molecular weight, amino acid composition and inhibition studies. The enzymes were inhibited by TLCK, DFP, and PMSF, indicating a role for histidine and serine in the catalytic mechanism. The enzymes also were inhibited by other known trypsin inhibitors, i.e., benzamidine, soybean trypsin inhibitor, leupeptin and antipain, but not by TPCK which is a chymotrypsin inhibitor. In addition, the enzymes were fractionated by benzamidine Sepharose-6B column chromatography, an affinity system for separation of trypsin-like enzymes. Furthermore, fish and bovine trypsin have optimum activity for synthetic substrates in the pH range of 9.0 to 10.0, but crawfish trypsins had maximal activity in the pH range of 7.5 to 8.0 which is comparable to other crustacea.

G. Substrate Specificities

The enzymatic activities of trypsins for synthetic and proteinaceous substrates are presented in Table IV-4. Purified

Table IV-4. Hydrolytic activities for synthetic and proteinaceous substrates by purified or crude crawfish hepatopancreatic trypsins

Substrate	Specific activity				
	A	B	C	D	Crude
TAME	870	1280	1250	970	16
BAEE	130	160	170	120	2.2
BAPNA	0.56	1.3	2.3	0.31	0.0036
ATEE	0	0	0	0	0.0027
BTEE	0	0	0	0	0.046
Casein	12	6.5	8.6	12	0.24
Myofibrillar proteins	7.9	4.4	5.5	8.0	0.17
Sarcoplasmic proteins	3.6	2.2	2.5	3.0	0.084
Bovine collagen	NM	NM	11	7.4	21

Specific activities for TAME, BAEE, BAPNA, ATEE, and BTEE are expressed with mM of substrate per mg protein per min at 45°C.

Specific activities for casein, myofibrillar and sarcoplasmic proteins are expressed with mM equilibrate tyrosine produced per mg protein per min at 45°C and pH 8.1.

TAME; N-Tosyl-L-arginine methyl ester

BAEE; Benzoyl-L-arginine ethyl ester

BAPNA; Benzoyl-D,L-arginine-p-nitroanilide

ATEE; N-Acetyl-L-tyrosine ethyl ester

BTEE; Benzoyl-L-tyrosine ethyl ester

NM; Not measured.

enzymes had higher esterolytic activity toward TAME than BAEE. Esterolytic activities of trypsin A, B, C, and D toward TAME were approximately 6.7, 8.0, 7.4, and 8.1-fold, respectively, higher than toward BAEE. Esterolytic activities for TAME and BAEE of purified trypsins were from 50 to 85-fold higher than crude enzyme activity; however, amidolytic activities of trypsin A, B, C, and D increased 150, 350, 640, and 86-times, respectively. Thus, highly different purities between esterolytic and amidolytic activities were detected in this study but the reason is not apparent. One possibility is that the crude enzyme, containing highly chromogenic organics, can interfere with the measurement of optical density, even though it was filtered through a microfilter.

No activity was detected with ATEE and BTEE, which are specific substrates for chymotrypsin. This indicates that the four enzymes are serine proteases and provides evidence that they are trypsin-like enzymes supporting results of the inhibitor studies. Although very poor chymotryptic activities against ATEE or BTEE were found in the crude enzyme of crawfish hepatopancreas, purified enzymes do not have chymotryptic activity. The removal of chymotrypsin could reduce caseinolytic activity, thus the caseinolytic activities of purified trypsin A, B, C, and D increased 50, 27, 36, and 50-fold, respectively, compared with crude enzyme. Increases of purities measured with caseinolytic activities were less than those with esterolytic and amidolytic activities. This

may have occurred because other proteolytic enzymes, including chymotrypsin, were removed through purification. Weak chymotrypsin activity was detected in the hepatopancreas of prawn, Macrobrachium rosenbergii (Lee et al., 1980; Baranowski et al., 1984). However, Lindner et al (1989) reported that homogenates of prawn hepatopancreas did not show chymotrypsin-like activity toward the synthetic substrate glutaryl-L-phenylalanine-p-nitroanilide (GPNA).

Proteolytic activities against crawfish myofibrillar and sarcoplasmic proteins were less than those against casein substrate. The level of proteolytic activities against casein, and myofibrillar and sarcoplasmic proteins was consistent among the trypsins. Mushiness in ice stored prawn is accompanied only by a limited degree of proteolysis of myofibrillar proteins due to collagenolytic enzymes diffused from the hepatopancreas (Lindner et al., 1988). Also, the proteolysis was not due to a proteolytic system endogenous to the muscle. Degradation of myofibrillar proteins increased rapidly during the salt-curing process of herring at neutral and weak alkaline pH where activity was caused by digestive enzymes diffusing from the remaining gut into the brine (Olafsdottir et al., 1985). Also, casein- and hemoglobin-hydrolysing activities are due mainly to thiol proteases with a minor contribution from trypsin-like activity (Lindner et al., 1989).

With respect to specific activity for proteinaceous

substrates, trypsin A and D had higher activity than trypsin B and C. Thus, trypsin A and D may be more responsible for development of mushiness in crawfish tail meat.

H. Degradation of Myofibrillar Proteins by Trypsin

SDS-PAGE was used to monitor the degradation of myofibrillar proteins by purified trypsin A from crawfish hepatopancreas. The degradation of myofibrillar proteins from crawfish tail meat are presented in Fig. IV-22 at zero time and 1, 2, 4, 8, 12, 20, 30, 45, 60 and 120 min incubation with trypsin A. As incubation progressed up to 12 min, myosin heavy chain, c-protein, and actinin degradation were apparent, accompanied by an increase in protein fragments with molecular weight of 50-90K. Up to 4 min incubation, tropomyosin and troponins were not affected by trypsin A. Incubation for 20 min rendered complete degradation of the myosin heavy chain and actinin. After 20 min incubation, newly formed protein fragments (50-90K), actin, tropomyosin, and troponins were hydrolyzed gradually.

Autolysis of fish tissue is most likely due to solubilization of muscle protein caused by either proteolytic enzymes released from the intestinal tract or proteinases in muscle cells. Tryptic activity accounted for about 70% of the total proteolytic activity responsible for solubilization in capelin muscle tissue (Aksnes and Brekken, 1988). Trypsin-like proteinase from crawfish is very similar to mammalian trypsins

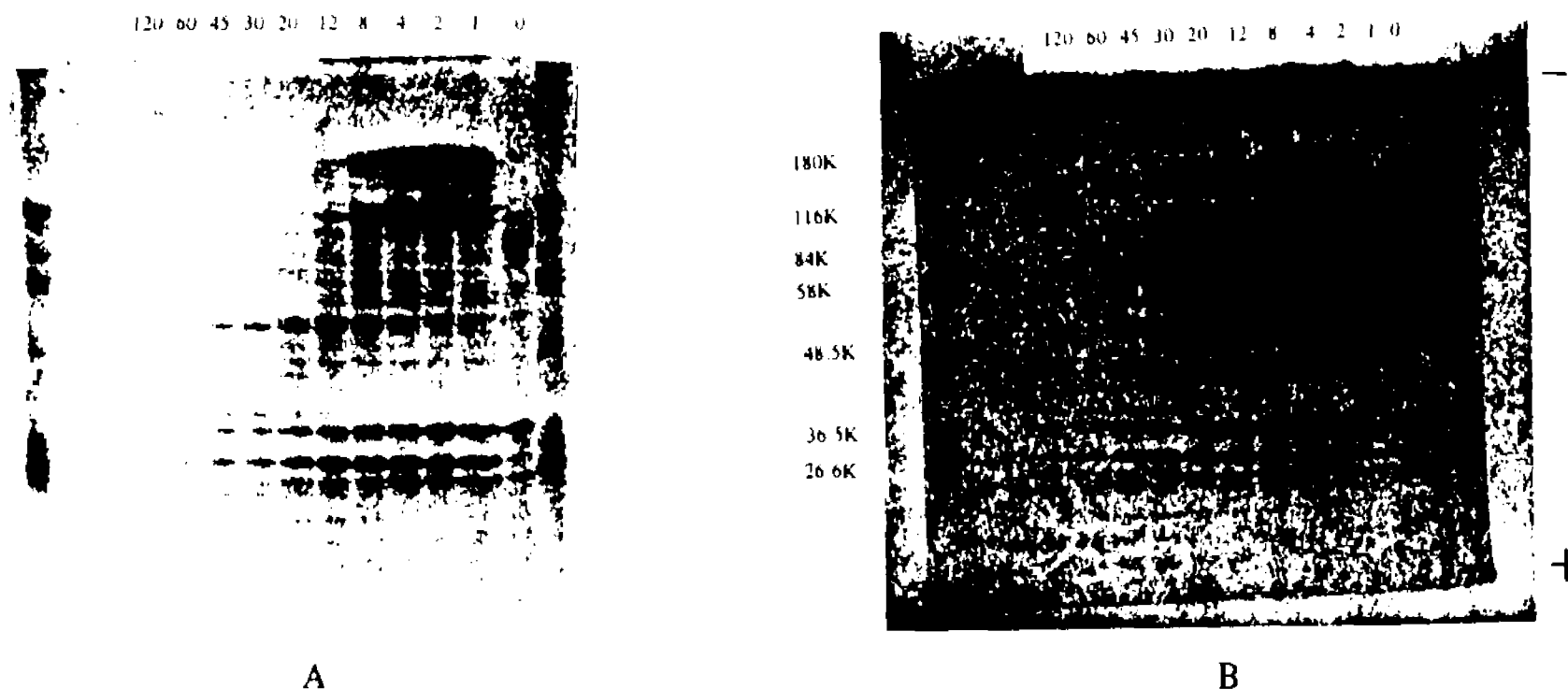


Fig. IV-22. SDS-PAGE analysis of the effect of incubation time on degradation of the myosin heavy chain from crawfish myofibrillar proteins. Digestion times (in minutes) are indicated under each lane. The molecular size of tryptic fragments is given in kilodaltons (K) on both sides of the gel. HC refers to the myosin heavy chain (200K), A corresponds to actin (45K). Standard proteins are α_2 -macroglobulin (180K), β -galactosidase (116K), fructose-6-phosphate kinase (84K), pyruvate kinase (58K), fumarase (48.5K), lactic dehydrogenase (36.5K), and triosephosphate isomerase (26.6K).

on the basis of their substrate specificity and inhibition studies. Limited tryptic digestion of myosin subfragment 1 leads to the formation of three stable peptides of 20K, 25K, and 50K fragments of S-1 (Mornet et al., 1981; Chen et al., 1987). The fragmentation of myosin by trypsin in rigor myofibrils was slow and led mainly to the formation of the 25K and 200K fragments (Lovell and Harrington, 1981). Further digestion of 200K heavy chain resulted in the formation of small amounts of LMM (80K) and HMM (130K and 140K) (Chen and Reisler, 1984). However, the degradation of myosin heavy chain by cathepsin D formed two major groups of fragments with molecular weight of 120K and 90K (Zeece and Katoh, 1989).

Textural degradation of oval-filefish meat gel accompanied by increased peptides and breakdown of myosin heavy chain was caused by proteinase associated with myofibrils (Toyohara et al., 1990). Myofibrillar proteins in fish meat gel were hydrolyzed and fish gel strength decreased by the addition of an edible mushroom (Makinodan and Hujita, 1990). These researches reported myofibrillar proteins, especially myosin, actin, and tropomyosin were degraded by proteinase from mushroom. Lysosomal cathepsins B and D are well known endopeptidases in cells with the potential of initiating the degradation of myofibrillar proteins (Bird et al., 1977). Salt-cured Icelandic herring has a particular taste and a soft consistency, which results largely from a partial degradation of the fish muscle by proteolytic enzymes

(Olafsdottir et al., 1985). Trypsin and hepatopancreatic homogenate caused a low level of mushiness, which was verified with SDS-PAGE patterns of myofibrillar proteins extracted from fresh prawns (Lindner et al., 1988).

EXPERIMENT 3. Kinetic and Thermodynamic Properties of Purified Trypsins

A. Kinetic Properties of Crawfish Hepatopancreatic Trypsins

The kinetic parameters (K_m' and V_{max}) for hydrolysis of p-tosyl-L-arginine methyl ester (TAME) by crawfish trypsin and bovine trypsin at pH 6.8 and 8.1 were examined with different substrate concentrations. Results obtained at 25°C are presented in Table IV-5 calculated from Lineweaver-Burk plots. The apparent Michaelis-Menten (K_m') constants and substrate turnover numbers of crawfish trypsin for the esterase reaction were similar at pH 6.8, but trypsin B had higher substrate turnover number (V_{max}). This indicates higher physiological efficiency compared to the other trypsin. However, the K_m' values of crawfish trypsin at pH 8.1 decreased compared to those at pH 6.8, which suggests higher affinity for TAME at alkaline pH.

Although crawfish trypsin had similar substrate turnover numbers for esterolytic reaction at pH 6.8, those at pH 8.1 were significantly higher, especially trypsin C, which increased about 1.8-fold. Increased turnover numbers and decreased K_m' values compared to pH 6.8 indicates higher physiological efficiencies (V_{max}/K_m) at pH 8.1.

The K_m' constants and turnover numbers of crawfish trypsin were higher than those for bovine trypsin at pH 6.8. Other workers have found that anionic trypsin from catfish had kinetic properties similar to those of bovine trypsin

Table IV-5. Kinetic properties of crawfish hepato-pancreatic and bovine trypsins for the hydrolysis of N-p-tosyl-L-arginine methyl ester (TAME)

pH	Enzyme	V_{max} (sec^{-1})	K_m (mM)	V_{max}/K_m ($\text{sec}^{-1} \text{mM}^{-1}$)
6.8	Trypsin A	150	0.42	360
	Trypsin B	230	0.41	570
	Trypsin C	170	0.42	400
	Trypsin D	160	0.39	410
	Bovine trypsin	100	0.33	300
8.1	Trypsin A	200	0.33	590
	Trypsin B	370	0.27	1,400
	Trypsin C	300	0.42	740
	Trypsin D	230	0.22	1,000
	Bovine trypsin	220	0.30	740

Reaction condition was 0.5 ml of various concentration of TAME and 25 μl of crawfish trypsins (10 $\mu\text{g}/\text{ml}$) at 25°C and pH 6.8 or 8.1.

Turnover number is TAME units per mM enzyme.

(Yoshinaka et al., 1984). Most intracellular enzymes normally function at substrate concentrations similar to or below their K_m values. However, the K_m' value of digestive enzymes may not be critical to their functionality because trypsin may be required to function at relatively high substrate concentration. Under these conditions, substrate turnover number could be a more important parameter. On the basis of this reasoning, crayfish trypsin B at pH 6.8 and trypsin B and C at pH 8.1 are probably better catalysts for hydrolysis of TAME than are the other enzymes.

The kinetic parameters (K_m' and V_{max}) for esterase reaction with TAME were thoroughly studied with trypsin from crayfish (Pfleiderer et al., 1967), bovine (Magalhaes-Rocha et al., 1980), carp (Cohen et al., 1981b), Greenland and Atlantic cod (Simpson and Haard, 1984b; Simpson et al., 1989), and Atlantic cod (Asgeirsson et al., 1989). Different K_m' values have been reported for bovine trypsin for TAME hydrolysis, i.e., 0.125 mM (Magalhaes-Rocha et al., 1980), 0.53 mM (Cohen et al., 1981b), and 0.05 mM (Simpson and Haard, 1984b). Differences may be due to different reaction condition such as pH, temperature, and concentration of enzymes and substrate. Cohen et al. (1981b) reported that kinetic properties of carp and mammalian trypsin were similar. However, they demonstrated that kinetic parameters for esterase reactions (TAME and BAEE) were different depending on substrate concentration, i.e., K_m' values of 0.013 and 0.307 mM, for carp trypsin with two

different substrate concentrations.

Table IV-6 presents results obtained from kinetic measurement at 45°C using benzoyl-D,L-arginine-p-nitroanilide (BAPNA). The apparent Michaelis-Menten (K_m') constants and substrate turnover numbers (V_{max}) of crawfish trypsins for the amidolytic reaction were different among enzymes at pH 6.8. Trypsins B and C had higher turnover numbers and lower K_m values compared with the other trypsins, which indicates higher physiological efficiencies. The K_m' values of crawfish trypsins for BAPNA hydrolysis were notably low compared with that of bovine trypsin at both pH's. The K_m values of trypsin from Greenland and Atlantic cod (1.67 and 1.48 mM, respectively) were found to be higher than those of bovine trypsin (Simpson and Haard, 1984b, Simpson et al., 1989); however, three trypsins from Atlantic cod were reported to be comparable to bovine trypsin with values of 0.077 to 0.102 mM (Asgeirsson et al., 1989). Thus, K_m values of enzymes are highly influenced by the concentration of substrate and enzyme concentration as well as by pH and temperature.

The K_m' values at pH 8.1 were much lower than those at pH 6.8 since amidase activities at pH 8.1 were inhibited by the substrate concentration. Therefore, increased affinities caused considerably higher physiological efficiencies at alkaline pH than at neutral pH. All of the enzymes had different catalytic effects for the amidolytic reaction at both pH points on the basis of substrate turnover number.

Table IV-6. Kinetic properties of crawfish hepato-pancreatic and bovine trypsins for the hydrolysis of benzoyl-D,L-arginine-p-nitroanilide (BAPNA)

pH	Enzyme	V_{max} (sec^{-1})	K_m (mM)	V_{max}/K_m ($\text{sec}^{-1} \text{mM}^{-1}$)
6.8	Trypsin A	0.31	0.43	0.73
	Trypsin B	0.58	0.23	2.6
	Trypsin C	0.86	0.13	6.9
	Trypsin D	0.17	0.31	0.56
	Bovine trypsin	0.20	0.64	0.30
8.1	Trypsin A	0.60	0.13	4.4
	Trypsin B	0.41	0.03	13
	Trypsin C	6.50	0.03	170
	Trypsin D	0.11	0.03	2.9
	Bovine trypsin	0.27	0.50	0.54

Reaction condition was 0.5 ml of various concentration of BAPNA and 25 μl of crawfish trypsins (100 $\mu\text{g}/\text{ml}$) at 45°C and pH 6.8 or 8.1.

Turnover number is BAPNA units per mM enzyme.

Moreover, the catalytic activity of trypsin C increased dramatically from pH 6.8 to 8.1. Differences in physiological efficiencies observed for crawfish trypsins at different pH were not apparent for bovine trypsin. Physiological efficiencies increased from pH 6.8 to 8.1 nearly 6 times for trypsin A, B, and D, and about 25 times for trypsin C. This supports the observation that trypsin C is the best catalyst for the amidolytic reaction at pH 8.1. The increased physiological efficiency is obtained through a decrease of K_m values rather than by an increase of substrate turnover numbers. The low K_m values at pH 8.1 obtained for crawfish trypsins are similar to values reported from carp trypsin (Cohen et al., 1981b), Antarctic krill trypsins (Osnes and Mohr, 1985b), and menhaden trypsin (Pyeun et al., 1990).

Although amidolytic activities were different from esterolytic activities, the kinetic results with both substrates evidenced similar patterns. Crawfish trypsins had considerably higher substrate affinities and higher turnover numbers for amidolytic reaction compared with bovine trypsin, as also occurred for esterase activity. However, crawfish trypsin C had extremely high catalytic activity for hydrolysis of BAPNA at both pH's compared with other trypsins and bovine trypsin. This was not the case in the esterolytic reaction.

Trypsins from crawfish hepatopancreas have higher affinities and catalytic activities toward ester substrate than amide substrate. This also has been reported for trypsin

from teleost fish (Cohen et al., 1981b; Simpson and Haard, 1984a; Martinez et al., 1988; Asgeirsson et al., 1989; Simpson et al., 1989), and in crustacea (Osnes and Mohr, 1985b; Honjo et al., 1990). Crawfish trypsin A, B, and D had higher turnover numbers for BAPNA hydrolysis than did bovine trypsin. Similarly, trypsin from Greenland cod had a much higher turnover number for BAPNA than bovine trypsin under conditions where substrate concentration was high enough to produce zero order kinetics (Simpson and Haard, 1984b). However, these researchers reported that the physiological efficiency of cod trypsin could be lower than that of bovine enzyme at certain temperatures and under conditions of low substrate concentration.

The ratio between turnover number for amidase and esterase was higher for trypsin B and C at pH 6.8 and trypsin A and C at pH 8.1 than was bovine trypsin (Table IV-7). Trypsin C had much higher amidolytic activity compared with other trypsins at both pH. The ratio between physiological efficiency for amidase and esterase reactions was considerably higher for crawfish trypsins than for bovine trypsin. Higher physiological efficiencies with amide substrates compared with bovine trypsin have been found in trypsin from carp (Cohen et al., 1981b), Greenland cod (Simpson and Haard, 1984b), anchovy (Martinez et al., 1988), and Atlantic cod (Simpson et al., 1989; Asgeirsson et., 1989). The hydrolytic function of trypsin is to attack the carboxyl side of arginine and lysine

Table IV-7. Comparison of amidase and esterase activities of crawfish hepatopancreatic trypsins at different pH

Enzyme	Turnover number amidase/esterase($\times 10^{-3}$)		Physiological efficiency amidase/esterase($\times 10^{-3}$)	
	pH 6.8	pH 8.1	pH 6.8	pH 8.1
Trypsin A	2.1	3.0	2.0	7.4
Trypsin B	2.5	1.1	4.5	9.3
Trypsin C	5.1	21	17	230
Trypsin D	1.1	0.47	1.4	2.8
Bovine trypsin	1.9	1.2	1.0	0.72

Turnover number is BAPNA or TAME units per mM trypsin. Catalytic efficiency is turnover number divided by K_m at the given pH. Data are average values of triple determinations.

residues in peptide bonds. Due to the close relationship between amides of the α -carboxyl groups and the corresponding peptides, Mihalyi (1978) suggested that amide substrates such as BAPNA are more relevant to the hydrolysis of peptide bonds than are ester bonds. Thus, fish trypsins are more suitable for hydrolysis of BAPNA than bovine trypsin. However, activities of crawfish trypsins against protein hydrolysis compare better with TAME activities than BAPNA activities. Hara et al. (1984) reported that K_m' values of two proteinases from rotifer for casein hydrolysis were the reverse of K_m' value for BAPNA hydrolysis, which is in good agreement with the crawfish trypsins.

Casein hydrolysis kinetics at 45°C are presented in Table IV-8 calculated by linear regression of the Lineweaver-Burk plot. Apparent Michaelis-Menten (K_m') constants of crawfish trypsins were similar among crawfish enzymes, and all were similar to bovine trypsin at both pH points. Crawfish trypsin B had the highest affinity (lowest K_m) against casein at pH 6.8, which resulted in the highest physiological efficiency at that pH. Trypsin B and C had lower turnover number for the hydrolysis of casein compared with other trypsins at both pH's, which is the opposite of results for BAPNA. The apparent Michaelis-Menten (K_m') constants at pH 8.1 were variable among the enzymes, with trypsin A and B having higher values than at pH 6.8. Bovine trypsin had greatest substrate affinity and turnover number among trypsins at pH 8.1 indicating greatest

Table IV-8. Kinetic properties of crawfish hepato-pancreatic and bovine trypsins for the hydrolysis of casein

pH	Source	V_{max} (sec^{-1})	K_m (%)	V_{max}/K_m ($\%^{-1}\text{sec}^{-1}$)
6.8	Enzyme A	5.5	0.32	17
	Enzyme B	3.3	0.17	19
	Enzyme C	3.3	0.40	8.3
	Enzyme D	6.9	0.39	18
	Bovine trypsin	4.4	0.33	13
8.1	Enzyme A	7.9	0.56	14
	Enzyme B	4.1	0.34	12
	Enzyme C	3.4	0.36	9.5
	Enzyme D	5.7	0.33	17
	Bovine trypsin	15	0.29	52

Reaction condition was 0.25 ml of various concentration of casein and 50 μl of crawfish trypsin (100 $\mu\text{g}/\text{ml}$) at 45°C and pH 6.8 or 8.1.

Turnover number is tyrosine equilibrate units per mM enzyme.

physiological efficiency among enzymes. A notable feature was that trypsin C had lower substrate turnover number and the lowest physiological efficiency among the trypsins at both pH points, which is in contrast to data from the amidolytic reaction. Crawfish trypsin A and D had a higher turnover number at pH 6.8 compared with other enzymes. This is consistent with results from the effect of pH on the hydrolysis of casein. However, trypsin A and D had higher turnover number at pH 8.1 than the other crawfish trypsins, although considerably lower than bovine trypsin. The higher K_m' values and the lower turnover number of crawfish trypsins for casein hydrolysis induced lower physiological efficiencies than bovine trypsin at pH 8.1. In contrast, crawfish trypsins had greater proteolytic activities than bovine trypsin at physiological pH (6.8). Trypsin D had the highest substrate turnover number followed by trypsin A compared with the other trypsins at pH 6.8. Thus, these two enzymes may play a primary role in the development of mushiness in crawfish tail meat compared with other trypsins. Moreover, trypsin D may greatly contribute to mushiness in blanched crawfish tail meat due to its higher thermostability.

K_m' value for casein with bonefish crude protease (Jany, 1976) was 4.2×10^{-4} at 25°C and pH 9.0, which indicates that crawfish trypsins have lower affinity for casein than do bonefish trypsins. Also, Hara et al. (1984) reported low K_m' values for casein with two proteinases from rotifer, i.e., 4.4

$\times 10^{-2}$ and 5.2×10^{-2} , and K_m' value for BAPNA were the reverse of results for casein hydrolysis. Caseinolytic activities of crawfish trypsins were lower than alkaline proteinases from mackerel (Kim and Pyeun, 1986) and skipjack (Pyeun et al., 1988). However, Greenland cod and porcine pepsins have higher K_m' values and turnover numbers than crawfish trypsin (Squires et al., 1986). The K_m' value of alkaline proteinases in the pyloric caeca of mackerel was determined at different temperature and the value increased with increased reaction temperature (Ooshiro et al., 1971). Also, the K_m' value for hemoglobin by Greenland cod pepsin increased with increased temperature and was considerably higher than porcine pepsin (Squires et al., 1986).

The alimentary tracts of vertebrates have an acidic stomach, which secretes pepsin to hydrolyze food proteins prior to intestinal trypsins and other endopeptidases. Crustacea lack a digestive function similar to that of the acidic stomach (Lu et al., 1990). Therefore, it is important that the crawfish trypsins hydrolyze native protein more effectively than vertebrate trypsin at physiological condition. These results reveal the role of crawfish trypsins in digestive function as well as their potential involvement in the development of mushiness in crawfish tail meat.

The Kinetic parameters (K_m' and V_{max}) for hydrolysis of p-benzoyl-L-arginine ethyl ester (BAEE) were examined at 25°C for crawfish trypsins and bovine trypsin at pH 6.8. The

results obtained are presented in Table IV-9 calculated by linear regression of Lineweaver-Burk plots. Apparent Michaelis-Menten (K_m') constants and substrate turnover numbers of crawfish trypsins for hydrolysis of BAEE were different among the enzymes, with trypsin B and C having higher turnover number, similar to results for the amidolytic reaction. The esterolytic activities for the BAEE were low with the same substrate concentration applied for TAME reaction. Thus, the apparent K_m' values for BAEE were significantly lower than for TAME, which indicates higher affinity and physiological efficiencies. The K_m' values of crawfish trypsins for BAEE hydrolysis also varied among the enzymes. Reeck and Neurath (1972) reported that specific activities of lungfish and dogfish trypsin toward BAEE were similar compared with bovine trypsin. Also, trypsin from Streptomyces griseus had a K_m' value for BAEE similar to bovine trypsin (Olafson and Smillie, 1975), which differs from the results of the present experiment. Trypsin B, where turnover number was 1.8-fold higher than bovine trypsin, would be the best catalyst for the hydrolysis of BAEE on the basis of turnover number, but its physiological efficiency was less than that of bovine trypsin. The higher physiological efficiency of bovine trypsin is due to a considerably lower K_m' value rather than to a higher substrate turnover number. The kinetic results for BAEE hydrolysis differed from TAME hydrolysis, despite the fact that both substrates represent

Table IV-9. Kinetic properties of crawfish hepato-pancreatic and bovine trypsins for the hydrolysis of benzoyl-L-arginine ethyl ester (BAEE)

Source	V_{max} (sec^{-1})	K_m (mM)	V_{max}/K_m ($\text{mM}^{-1}\text{sec}^{-1}$)
Crawfish			
Enzyme A	56	0.03	2,000
Enzyme B	91	0.07	1,300
Enzyme C	78	0.09	830
Enzyme D	49	0.02	2,800
Bovine trypsin	53	0.03	1,600

Reaction condition was 0.5 ml of various concentration of BAEE and 25 μl of crawfish trypsins (10 $\mu\text{g}/\text{ml}$) at 25°C and pH 8.1.

Turnover number is BAEE units per mM enzyme.

esterolytic reactions. Trypsin C had lowest physiological efficiency among trypsins, whereas, it was found to be the most efficient catalyst for hydrolysis of BAPNA, as reported previously.

B. Thermodynamic Properties of Crawfish Trypsins

Activation energies for hydrolysis of TAME, BAEE, BAPNA, and casein by crawfish trypsins are summarized in Table IV-10. The esterolytic reaction using TAME or BAEE had similar activation energies. The crawfish trypsins had activation energies around 6.4 to 9.0 Kcal/mole, which were considerably lower than those for bovine trypsin (Simpson and Haard, 1984b). Results were in agreement with other fish trypsins where activation energies of 6.8 to 7.8 Kcal/mole have been reported for three trypsin from antarctic krill (Osnes and Mohr, 1985b), 8.5 Kcal/mole for Greenland cod (Simpson and Haard, 1984b), and 8.95 Kcal/mole for Greenland cod (Asgeirsson et al., 1989).

The activation energies for hydrolysis of BAPNA ranged from 5.8 to 6.2 Kcal/mole and were considerably lower than the esterolytic reaction. Also, activation energies of trypsins from anchovy intestine were estimated by extrapolation of the Arrhenius plots and were found to be 6.43 Kcal/mole for trypsin A and 5.95 Kcal/mole for trypsin B. This range has been reported for trypsin in Greenland cod (Simpson and Haard, 1984b) and anchovy (Martinez et al., 1988). However, higher

Table IV-10. Thermodynamic free energy of activation values for trypsin catalyzed hydrolysis of N-p-tosyl-arginine methyl ester (TAME), benzoyl-D,L-arginine-p-nitroanilide (BAPNA), and casein at pH 6.8

Enzyme	E _a (Kcal/mol)			
	TAME	BAEE	BAPNA	Casein
Trypsin A	6.8	6.7	6.9	13
Trypsin B	6.4	6.6	6.2	11
Trypsin C	9.0	8.2	5.9	9.8
Trypsin D	7.2	9.4	6.1	12
Bovine ¹	13	NM	12.7	11

Data are average values of triple determination. Temperature range over which Arrhenius experiment was done as follows; TAME, BAEE and BAPNA in 25-45°C and casein in 30-45°C.

¹ data from Simpson and Haard (1984b).

activation energies were reported for rainbow trout (McLeese and Stevens, 1982) and Atlantic cod (Simpson and Haard, 1989; Asgeirsson et al., 1989). Activation energies for the hydrolysis of casein varied among trypsins with trypsin D similar to bovine trypsin.

EXPERIMENT 4. Utilization of Crawfish Hepatopancreas

A. Proximate Composition of Whole Crab, Claw Waste, and Crab Mince

The proximate composition of crab waste is given in Table IV-11. Moisture and protein content of shell waste (SW) were lower than mince, but ash content, which consisted mostly of calcium, was higher than mince. Protein content of SW was relatively high due to the presence of heat-denatured proteins. Moreover, a subjective evaluation of crab flavor indicated higher levels in SW than in mince. The presence of high levels of volatile crab flavors and 14% protein in SW suggests its potential as a crab flavor source. Therefore, SW was used for subsequent studies on the extraction of volatile flavor compounds through incorporation of enzyme from crawfish hepatopancreas.

B. Optimum Conditions for Protein Hydrolysis of Crab Waste Using Crude Enzyme Extract of Crawfish Hepatopancreas

1) Effect of pH on Hydrolysis of Crab Waste Protein

The effect of pH on hydrolysis of crab waste protein by menhaden and crawfish enzymes is illustrated in Table IV-12. Crawfish crude enzyme had lower protein hydrolytic activity than did menhaden enzyme. Hydrolytic activities were highest at pH 9.0 (0.1 M NaHCO_3 - Na_2CO_3) for both enzymes. The hydrolytic activity was different among the buffer solution

Table IV-11. Approximate composition of whole and claw waste and minced meat of crab. Parentheses indicate standard error

Sample	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
HPW	59.5 (0.98)	14.0 (0.18)	0.3 (0.01)	19.6 (0.83)
HPC	48.3 (0.83)	14.7 (0.18)	0.2 (0.02)	32.4 (0.37)
WM	78.1 (0.05)	18.9 (0.06)	0.6 (0.01)	3.8 (0.09)

HPW and HPC: hard-tissue waste of whole and claw of crab waste, respectively.

WM: mince obtained from whole crab waste.

Table IV-12. Effect of enzyme concentration on the release of total amino compounds

Treatment	Amino cpds (Tyr.eq. mM)	Prot.conc. (mg/ml)	Transmittance (% at 450 nm)
Control (without enzyme)	1.29	1.10	5.43
0.5 ml of enzyme	6.97	0.18	7.84
1 ml of enzyme	8.53	0.07	15.63
2 ml of enzyme	7.80	0.07	18.19

Reaction was performed at pH 9.0 and at 45°C for 4 hr.

species, with lower activity for both enzymes in Tris-HCl buffer solution compared with other solutions. This result was similar to that found with caseinolytic activity (Fig. IV-13). Therefore, hydrolysis of crab waste protein by crawfish enzyme was performed at pH 9.0.

2) Effect of Enzyme Concentration and Incubation Time on Crab Waste Protein Hydrolysis

The degree of hydrolysis of crab waste protein by different concentrations of crawfish crude enzyme solution is indicated in Table IV-13. Amino compounds significantly increased due to addition of enzyme solution, and protein decreased as a result of hydrolytic activity. Amino compounds produced from degradation of waste protein were higher with 1 ml of enzyme than with 2 ml of enzyme. This may be due to product inhibition by excessive low molecular weight of protein hydrolysates. Neito et al. (1990) reported that increasing chymotrypsin concentration increased rate of solubilization of crab meat but not total conversion.

Table IV-14 shows the effect of incubation time on the hydrolysis of crab waste protein at pH 9.0 and 45°C with 0.5 ml of enzyme solution. Amino compounds increased with reaction time and protein decreased due to hydrolytic action. Further increase in amino compounds was slight after 2 hr reaction time compared with shorter incubation periods. Also, the clarity, i.e., low transmittance, of waste hydrolysate

Table IV-13. Effect of incubation time on the hydrolysis of waste protein

Incubation time (hr)	Amino cpds (Tyr.eq. mM)	Prot.conc. (mg/ml)	Transmittance (% at 450 nm)
0 hr	1.07	0.82	16.99
0.5 hr	2.64	0.32	41.34
1 hr	4.23	0.23	40.50
2 hr	6.08	0.15	39.64
4 hr	7.04	0.11	39.45

Reaction was performed at pH 9.6 (0.05 M Na_2CO_3 - NaHCO_3) and at 45°C. Reaction was performed with 0.5 ml of crawfish crude enzyme.

Table IV-14. Effect of pH on the hydrolysis of waste protein. Reaction was performed at 45°C for 2 hr

pH	Amino cpds (Tyr.eq. mM)		Prot.conc. (mg/ml)	
	Menhaden	Crawfish	Menhaden	Crawfish
Control (Without enzyme)				
	1.85	1.85	1.02	1.02
0.1 M Phosphate buffer				
6.0	6.46	4.55	0.19	0.97
6.5	6.83	3.94	0.26	0.49
7.0	6.83	4.12	0.26	0.64
7.5	6.83	5.32	0.26	0.73
8.0	7.26	6.34	0.33	0.75
0.1 M Tris-HCl buffer				
8.0	6.95	4.55	0.14	0.25
8.5	6.95	5.07	0.16	0.71
9.0	7.38	4.67	0.17	0.25
0.1 M NaHCO_3 - Na_2CO_3 buffer				
9.0	7.53	6.02	0.22	0.48
9.5	7.20	5.35	0.24	0.66
10.0	7.01	5.35	0.33	0.55
10.5	6.97	4.80	0.33	0.90

Enzyme concentration was 0.85 mg/ml for menhaden and 8.0 mg/ml for crawfish crude enzyme solution. Reaction mixture was 5 g of crab waste, 7.5 ml of 0.1 M buffer solution and 0.5 ml of enzyme solution. Reaction was performed at 45°C for 2 hr at indicated pH.

solution was improved due to enzymatic hydrolysis.

C. Volatile Flavor Analysis of Enzyme Treated and Untreated Crab Waste

Total ion chromatograms of enzyme treated and untreated crab waste are presented in Fig IV-23a and IV-23b, respectively. One hundred seventeen compounds were identified and included 19 aldehydes, 29 aromatic hydrocarbons, 19 ketones, 10 alcohols, 12 nitrogen-containing compounds, 7 sulfur-containing compounds, and 16 miscellaneous compounds (Table IV-15).

Higher peak areas were obtained for enzyme treated crab waste than for untreated crab waste except for alkylbenzenes, dienal, octenal, and heptenal. The increase of volatile compounds in enzyme treated crab waste may be due to the diversity of enzymes distributed in crawfish hepatopancreas. These may facilitate the extraction of volatile components entrapped in crab waste by degradation of muscle proteins or increase flavor precursors, i.e., amino acids and aldehydes, for generation of volatile compounds during thermal extraction and distillation.

1) Aldehydes

Twenty aldehydes were identified in crab waste and 19 aldehydes were detected in the enzyme treated crab waste. (E,Z)-2,4-Decadienal was not detected in enzyme treated crab waste. An interesting pattern was noted for ratios of

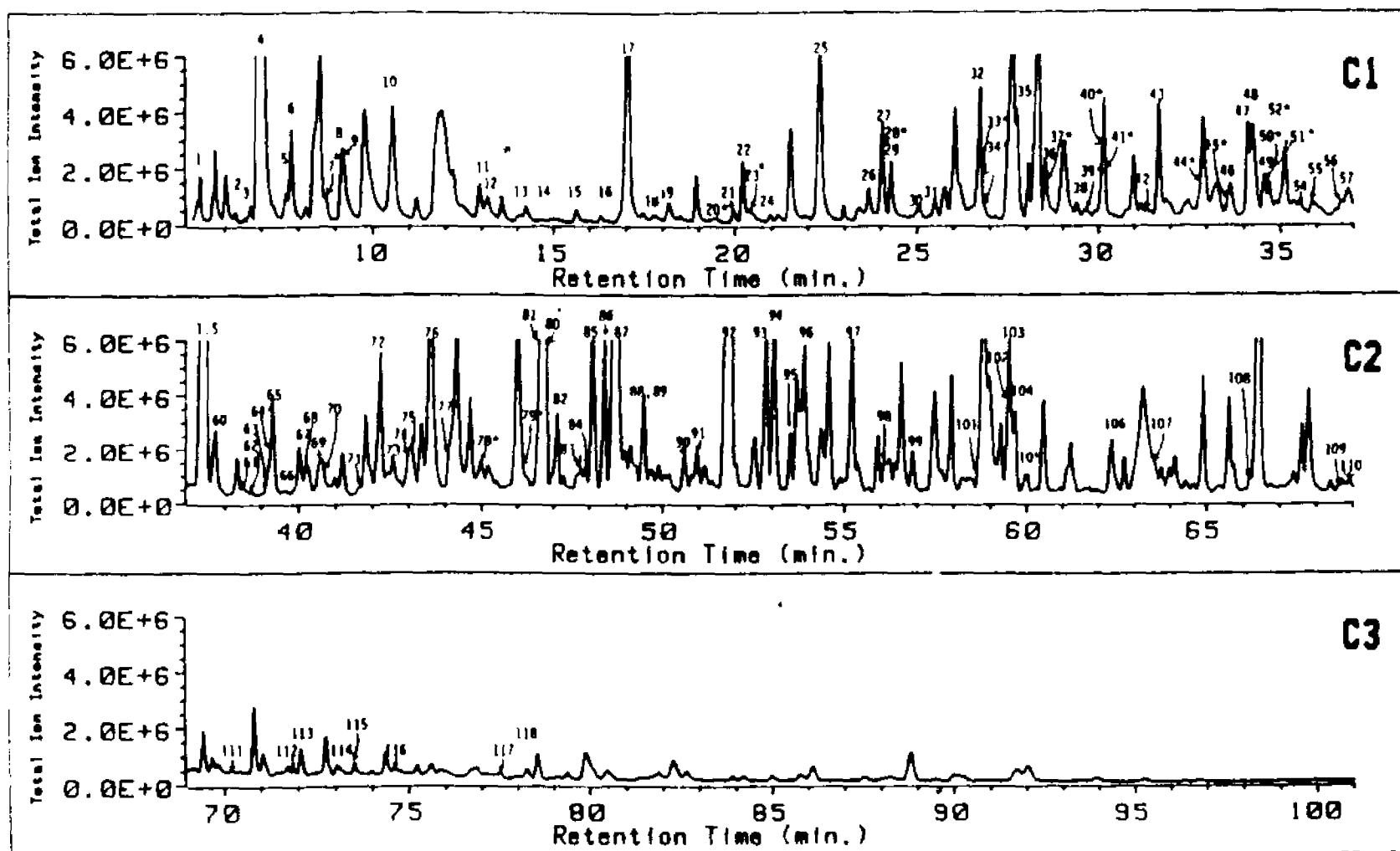


Fig. IV-23a. Total ion chromatogram of volatile flavor components from the simultaneous distillation and extraction of nontreated crab waste. Peak numbers correspond to those shown in Table IV-15.

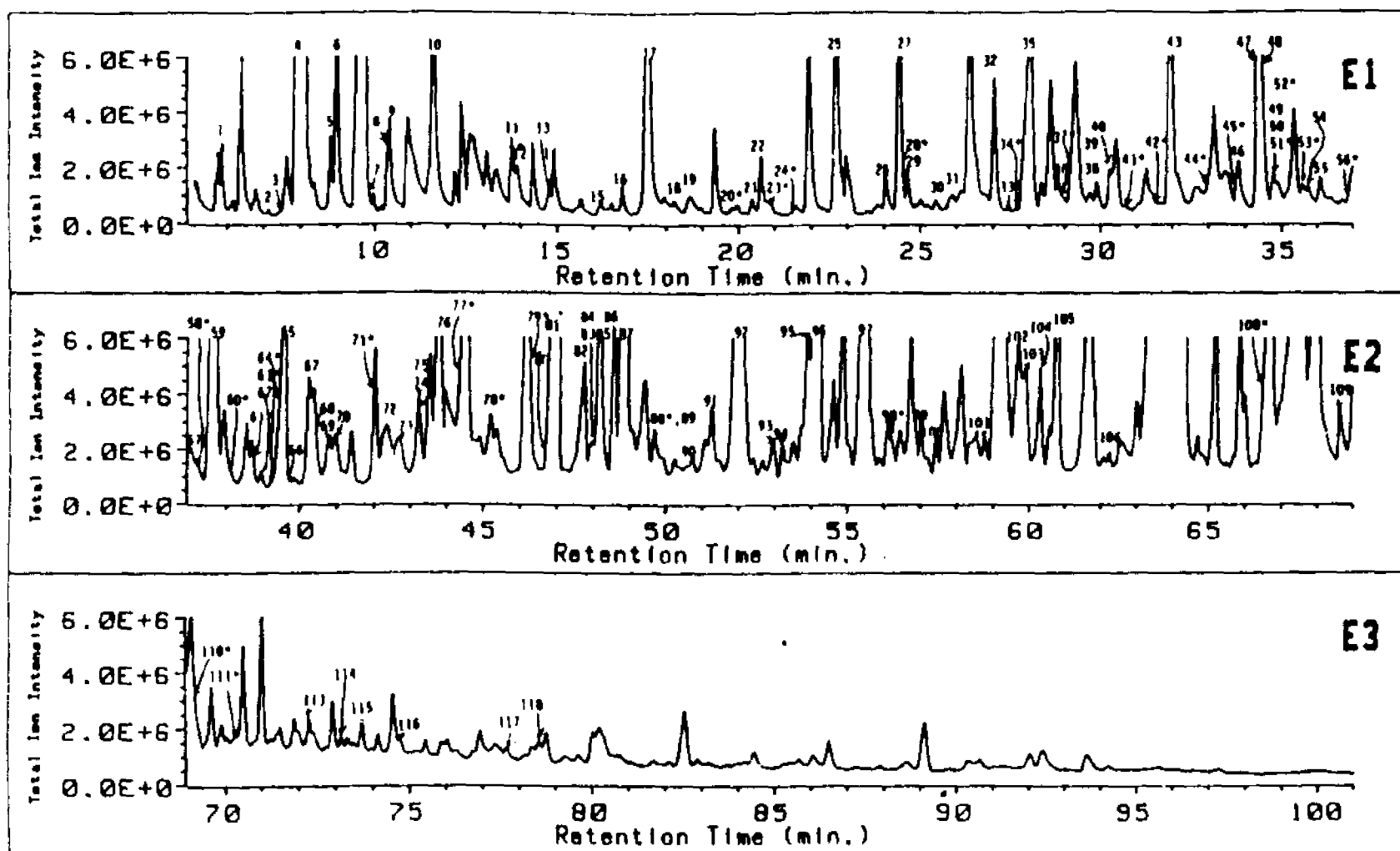


Fig. IV-23b. Total ion chromatogram of volatile flavor components from the simultaneous distillation and extraction of enzyme treated crab waste. Peak numbers correspond to those shown in

Table IV-15. Comparison of volatile components from enzyme treated (E) and nontreated crab waste (C)

Compounds		RI	Avg. (E)		SD	Avg. (C)		SD	Ratio
Aldehydes									
5	2-Methylbutanal	917	0.0954	0.0093		0.0410	0.0129		2.33 ³
6	3-Methylbutanal	921	0.3221	0.0328		0.1391	0.0344		2.32 ³
10	Pentanal	982	0.5260	0.0358		0.2854	0.0478		1.84 ³
14	(E)-2-Butenal	1040	0.0851	0.0128		0.0307	0.0034		2.77 ³
17	Hexanal	1082	0.7774	0.1750		0.3997	0.0681		1.95
18	2-Methyl-(E)-2-butenal	1094	0.0274	0.0049		0.0140	0.0082		1.96
22	(E)-2-Pentenal	1129	0.0896	0.0114		0.0707	0.0208		1.27
29	Heptanal ²	1184	0.0285	0.0083		0.0266	0.0047		1.07
32	(E)-2-Hexenal	1218	0.2117	0.0155		0.1916	0.0384		1.10
36	(Z)-4-Heptenal	1241	0.0962	0.0276		0.1119	0.0170		0.86
49	(E)-2-Heptenal	1323	0.0077	0.0014		0.0098	0.0016		0.79
66	Nonanal ²	1392	0.0017	0.0005		0.0062	0.0041		0.27
68	(E,E)-2,4-Hexadienal ²	1402	0.0066	0.0018		0.0096	0.0021		0.69
72	(E)-2-Octenal	1429	0.2500	0.0232		0.2218	0.0227		1.13
80	(E,E)-2,4-Heptadienal ²	1494	0.1954	0.0680		0.6442	0.0834		0.30 ³
87	Benzaldehyde	1526	0.7061	0.0243		0.5009	0.0362		1.41 ³
88	(E)-2-Nonenal ²	1538	0.0143	0.0038		0.0290	0.0088		0.50
93	(E,Z)-2,6-Nonadienal ²	1589	0.0396	0.0262		0.1060	0.0283		0.37 ³
94	(E,E)-2,4-Octadienal ^{1,2}	1592	0.0759	0.0267		0.2282	0.0273		0.33 ³
107	(E,Z)-2,4-Decadienal ¹	1768	ND			0.0207	0.0048		- ³
Alcohols									
12	Butanol ²	1024	0.1329	0.0109		0.0909	0.0187		1.46 ³
25	1-Penten-3-ol ²	1158	0.4013	0.0250		0.3368	0.0480		1.19
46	(Z)-2-Penten-1-ol	1309	0.0886	0.0165		0.0602	0.0113		1.47
48	(E)-2-Penten-1-ol	1318	0.3397	0.0495		0.1905	0.0136		1.78 ³
57	Hexanol ²	1352	0.0593	0.0118		0.0376	0.0016		1.58 ³
76	1-Octen-3-ol	1449	0.5978	0.0541		0.4217	0.0310		1.42 ³
91	Octanol ²	1559	0.0556	0.0061		0.0268	0.0032		2.08 ³
98	2-Methoxyphenol ^{1,2}	1640	0.0189	0.0008		0.0097	0.0005		1.95 ³
100	2-Furanmethanol ^{1,2}	1660	0.0194	0.0031		ND			- ³

Table IV-15 (continued)

111	Benzenemethanol ^{1,2}	1877	0.0078	0.0019	0.0028	0.0004	2.74 ³
Ketones							
8	2,4-Dimethylcyclobutanone ²	952	0.1900	0.0188	0.2937	0.1050	0.65
15	2,3-Pentanedione	1061	0.0269	0.0055	0.0197	0.0052	1.36
21	3-Penten-2-one	1125	0.0233	0.0012	0.0163	0.0044	1.43
27	2-Heptanone	1182	0.3109	0.0182	0.1213	0.0186	2.56 ³
38	3-Octanone	1253	0.0327	0.0044	0.0229	0.0010	1.43 ³
43	2-Octanone	1284	0.4239	0.0178	0.1629	0.0124	2.60 ³
54	6-Methyl-5-hepten-2-one	1336	0.0458	0.0078	0.0150	0.0150	3.06 ³
65	2-Nonanone	1389	0.4772	0.0296	0.1741	0.0129	2.74 ³
69	3-Octen-2-one	1406	0.0190	0.0007	0.0098	0.0008	1.93 ³
81	2-Decanone ²	1496	0.8173	0.0780	0.2335	0.0302	3.50 ³
84	3-Nonen-2-one	1513	0.0384	0.0384	0.0357	0.0058	1.08
86	(E,E)-3,5-Octadien-2-one ¹	1521	0.3005	0.0138	0.2071	0.0127	1.45 ³
92	(E,E)-3,5-Octadien-2-one ¹	1574	1.7787	0.1022	1.1635	0.0751	1.53 ³
95	Undecanone ²	1602	0.1743	0.0385	0.0682	0.0143	2.56 ³
99	1-Phenylethanone ^{1,2}	1653	0.0616	0.0028	0.0398	0.0014	1.55 ³
105	2-Dodecanone	1711	0.0575	0.0219	0.0267	0.0137	2.15
108	Tridecanone ²	1812	0.0594	0.0262	0.0255	0.0177	2.33
113	Tetradecanone ²	1915	0.0434	0.0126	0.0238	0.0117	1.82
118	Pentadecanone ²	2017	0.0240	0.0024	0.0120	0.0032	2.01 ³
Aromatic Hydrocarbons							
7	Benzene ²	941	0.0051	0.0007	0.0048	0.0025	1.05
13	Toluene ²	1038	0.0126	0.0016	0.0059	0.0001	2.16 ³
20	Ethylbenzene ²	1125	0.0013	0.0002	0.0038	0.0010	0.35 ³
23	p-Xylene	1132	0.0004	0.0001	0.0031	0.0009	0.12 ³
24	m-Xylene	1139	0.0016	0.0001	0.0088	0.0027	0.18 ³
28	o-Xylene	1182	0.0005	0.0002	0.0030	0.0008	0.16 ³
33	4-Ethyltoluene	1221	0.0007	0.0001	0.0018	0.0004	0.40 ³
34	3-Ethyltoluene	1223	0.0016	0.0002	0.0041	0.0008	0.40 ³
37	1,3,5-Trimethylbenzene	1242	0.0011	0.0003	0.0030	0.0006	0.38 ³
39	2-Ethyltoluene	1259	0.0006	0.0001	0.0017	0.0004	0.37 ³
41	p-Cymene	1268	0.0009	0.0001	0.0013	0.0003	0.70

Table IV-15 (continued)

42	1,2,4-Trimethylbenzene	1279	0.0048	0.0002	0.0089	0.0016	0.54 ³
44	C4-Alkylbenzene ¹	1299	0.0016	0.0003	0.0026	0.0005	0.60 ³
45	C4-Alkylbenzene ¹	1308	0.0008	0.0001	0.0012	0.0003	0.70
51	C4-Alkylbenzene ¹	1323	0.0074	0.0003	0.0124	0.0015	0.60 ³
53	1,2,3-Trimethylbenzene	1334	0.0075	0.0011	0.0139	0.0022	0.54 ³
56	C4-Alkylbenzene ¹	1351	0.0069	0.0003	0.0124	0.0018	0.56 ³
58	C4-Alkylbenzene ¹	1358	0.0094	0.0007	0.0166	0.0023	0.57 ³
60	C4-Alkylbenzene ¹	1366	0.0211	0.0006	0.0352	0.0056	0.60 ³
63	C4-Alkylbenzene ¹	1384	0.0011	0.0002	0.0020	0.0003	0.57 ³
71	C4-Alkylbenzene	1424	0.0144	0.0003	0.0278	0.0025	0.52 ³
73	1,2,4,5-Tetramethylbenzene ²	1435	0.0306	0.0007	0.0549	0.0031	0.56 ³
75	1,4-Dichlorobenzene ²	1443	0.0324	0.0018	0.0366	0.0036	0.89
79	C4-Alkylbenzene ¹	1488	0.0097	0.0005	0.0183	0.0011	0.53 ³
106	Naphthalene ²	1744	0.0719	0.0049	0.0821	0.0054	0.88
110	Methylnaphthalene ²	1857	0.0068	0.0015	0.0076	0.0016	0.89
112	Dimethylphenol ^{1,2}	1910	ND		0.0049	0.0010	- ³
114	Benzeneacetonitrile ^{1,2}	1930	0.0178	0.0018	0.0125	0.0027	1.43 ³
117	Phenol	2006	0.0243	0.0053	0.0168	0.0065	1.45
Nitrogen-containing Compounds							
26	Pyridine	1176	0.0650	0.0101	0.0633	0.0180	1.03
40	Methylpyrazine ²	1261	0.0622	0.0128	0.0482	0.0060	1.29
47	2,5-Dimethylpyrazine ²	1317	0.1332	0.0049	0.0972	0.0160	1.37 ³
50	2,6-Dimethylpyrazine ²	1323	0.0194	0.0011	0.0066	0.0012	2.95 ³
52	Ethylpyrazine ²	1330	0.0173	0.0011	0.0054	0.0008	3.20 ³
55	2,3-Dimethylpyrazine ²	1341	0.0134	0.0012	0.0029	0.0006	4.59 ³
62	2-Ethyl-6-methylpyrazine ²	1380	0.0222	0.0016	0.0053	0.0008	4.16 ³
64	2-Ethyl-5-methylpyrazine ²	1386	0.0292	0.0012	0.0225	0.0030	1.30 ³
67	Trimethylpyrazine ²	1398	0.0804	0.0025	0.0353	0.0058	2.28 ³
70	5-Ethyl-2-methylpyridine	1408	0.0390	0.0073	0.0249	0.0168	1.56 ³
74	2-Ethyl-3,6-dimethylpyrazine ²	1441	0.0333	0.0023	0.0272	0.0033	1.23
77	2-Ethyl-3,5-dimethylpyrazine ²	1456	0.0173	0.0014	0.0086	0.0014	2.01 ³
78	Tetramethylpyrazine ²	1470	0.0066	0.0008	0.0018	0.0004	3.62 ³
83	Dimethylpropylpyrazine ¹	1511	0.0493	0.0047	0.0357	0.0049	1.38 ³
85	Pyrrole	1516	0.4142	0.0630	0.2723	0.0227	1.52 ³

Table IV-15 (continued)

90	Methylpyrrole ²	1555	0.0282	0.0035	0.0125	0.0082	2.25
Sulfur-containing Compounds							
16	Dimethyl disulfide	1071	0.0244	0.0088	0.0102	0.0033	2.38
61	Dipropyl disulfide ²	1377	0.0008	0.0002	0.0023	0.0002	0.37 ³
96	3,5-Dimethyl 1,2,4-trithiolane	1607	0.4304	0.0208	0.1164	0.0297	3.70 ³
97	3,5-Dimethyl 1,2,4-trithiolane	1628	0.5283	0.0256	0.1465	0.0407	3.61 ³
101	3-Thiophenecarboxaldehyde ¹	1682	0.0172	0.0023	0.0187	0.0032	0.92
102	2-Thiophenecarboxaldehyde	1696	0.0526	0.0009	0.0556	0.0129	0.94
116	Benzothiazole	1958	0.0181	0.0037	0.0162	0.0041	1.12
Miscellaneous Compounds							
1	n-Octane	802	0.0426	0.0071	0.0318	0.0040	1.34
2	2-Octene ²	858	0.0046	0.0011	0.0072	0.0017	0.65
3	2-Methylfuran ²	873	0.0027	0.0008	0.0011	0.0006	2.45 ³
4	Ethyl acetate	901	2.2962	0.9047	1.3969	0.1057	1.64
9	2-Ethylfuran ²	954	0.0646	0.0078	0.0418	0.0133	1.55
11	Chloroform ²	1022	0.0925	0.0180	0.0530	0.0288	1.75
19	Undecane	1100	0.0429	0.0128	0.0346	0.0030	1.24
30	Limonene	1195	0.0231	0.0035	0.0246	0.0080	0.94
31	Dodecane ²	1200	0.0466	0.0028	0.0329	0.0055	1.42 ³
35	Pentylfuran ²	1231	0.4842	0.0567	0.2221	0.0676	2.18 ³
59	2,4,6-Trimethylpyridine(I.S.)	1362	1.0000	0.0000	1.0000	0.0000	1.00
82	Pentadecane	1508	0.2724	0.1178	0.1529	0.0908	1.78
89	Octylfuran	1538	0.0662	0.0080	0.0332	0.0031	1.99 ³
103	α -Terpineol ²	1699	0.0497	0.0091	0.0381	0.0090	1.30 ³
104	Heptadecane ²	1707	0.1873	0.0518	0.1121	0.0383	1.67
109	Hexanoic acid ²	1850	0.1253	0.0267	0.0158	0.0029	7.92 ³
115	β -Ionone ²	1939	0.0279	0.0108	0.0175	0.0112	1.60

¹: Tentative identification by matching a sample spectrum with literature reference spectra.

²: Peak area ratio of characteristic ion.

³: Significantly different between enzyme treated and nontreated crab waste, 95% confidence level.

ND: Not detected. I.S.: Internal standard.

aldehydes in enzyme treated crab waste compared with untreated crab waste. 6-C Aldehydes significantly increased in the enzyme treated crab waste. Alkanals and alkenals (except nonanal and (E)-2-nonenal) increased in the enzyme treated crab waste. However, all alkadienals detected in enzyme treated crab waste, i.e., (E,E)-2,4-hexadienal, (E,E)-2,4-heptadienal, (E,Z)-2,6-nonadienal, (E,E)-2,4-octadienal and (E,Z)-2,4-decadienal, decreased significantly. Alkadienals could serve as precursors for the formation of other volatile compounds. For example, 2,4-decadienal and cysteine can react to produce 3,5-dimethyl-1,2,4-trithiolanes (Ho et al., 1989). An increase of 3,5-dimethyl-1,2,4-trithiolane might have resulted in decreased 2,4-decadienal.

Polyunsaturated fatty acids are predominant in muscle and hepatopancreas of crab (Krzeczkowski et al., 1971; van der Horst et al., 1973; Chapelle, 1978; Pakrashi et al., 1989). Oxidized fish lipids could be characterized as painty or rancid. Certain volatile aldehydes resulting from the autoxidation of polyunsaturated fatty acids have appeared as the principal contributors to fish-like aroma (McGill et al., 1977; Josephson et al., 1983). Lipxygenases in crayfish hepatopancreas could degrade polyunsaturated fatty acids and result in formation of aldehydes.

Alkanals, alkenals, and alkadienals identified in crayfish may have been due to oxidation of polyunsaturated fatty acids (Bailey et al., 1989; Karahadian et al., 1989;

Hsieh et al., 1989). 3-Methylbutanal and pentanal have green, fruity, nutty, cheesy, or sweaty flavors, depending on concentration (Arctander, 1969).

Several eight- and nine-carbon aldehydes were major impact aroma compounds in fresh whitefish (Josephson et al., 1983) possibly formed by the action of lipxygenase. Hexanal was found in moderately fresh saltwater fish, but its formation may be the result of autoxidation rather than by enzyme-mediated reaction (Josephson and Lindsay, 1986). Kubota et al. (1986) reported that aldehydes were formed in boiled shrimp but were not detected in roasted shrimp. They concluded that aldehyde was easily produced in nonaqueous food systems.

Benzaldehyde, contributing an almond-like nutty and fruity aroma found in crayfish waste (Tanchotikul et al., 1989), crayfish tail meat (Vejaphan et al., 1988), and hepatopancreas (Hsieh et al., 1989), was abundant among the aldehydes in crab waste and its content increased due to enzyme treatment.

2) Alcohols

Ten alcohols were identified in the enzyme treated crab waste and nine in untreated crab waste. Most alcohols increased slightly with enzyme treatment, especially benzenemethanol, octanol and 2-methoxyphenol. 2-Furanmethanol was not detected in crab waste, which suggests that the compound was synthesized during enzyme treatment or precursors

were produced by enzyme and the compound formed during distillation.

Alcohols come mainly from oxidative decomposition of fats (Watanabe and Sato, 1971). Straight chain primary alcohols were found to have greenish, woody, and fatty flavor in general and were considered to play an important role in the overall flavor of boiled stew (Peterson and Chang, 1982). 1-Octen-3-ol, which contributes significantly to "mushroom" character and was most abundant in Shiitake mushroom (Chen et al., 1986), was observed in both treated and untreated crab waste.

Eight- and nine-carbon volatile alcohols were formed by the action of lipxygenases in mushrooms (Tressel et al., 1981), cucumber and melon fruits (Galliard and Phillips, 1976). Also, they contribute to a heavy, planty aroma in the overall fresh fish aroma blend (Josephson et al., 1983). Josephson et al. (1984) suggested formation of these compounds resulted from the action of lipxygenase in fresh fish.

3) Ketones

Nineteen ketones were identified in both enzyme treated and untreated crab waste. All ketone compounds increased with enzyme treatment except 2,4-dimethylcyclobutanone, which were lower after enzyme treatment. Enzyme treated to untreated ratios for 2-decanone, 6-methyl-5-hepten-2-one, 2-nonanone, 2-

octanone, undecanone and 2-heptanone were much higher than other ketones.

Generally lower threshold values of volatile ketones result in higher contributions to overall fresh fish-like odors (Josephson and Lindsay, 1986). Josephson and Lindsay (1986) suggested ketones might be produced by thermal oxidation degradation of polyunsaturated fatty acids rather than by the action of lipoxygenase.

Twelve ketones were identified in boiled crawfish tail meat, with 2,3-pentanedione having an intense buttery and desirable aroma (Hsieh et al., 1989). Two methylketones were identified in crab meat and were usually associated with green, fruity aroma and gave more floral aromas as chain length increased (Hsieh et al., 1989). Methyl ketones are produced from β -keto acids by heating after hydrolysis. 3,5-octadien-2-one contributing a fatty-fruity odor has been reported in roasted shrimp (Kubota et al., 1986) and meats (Greenberg, 1981). Several ketones were identified in shrimp (Kubota et al., 1986), crawfish tail meat (Vejaphan et al., 1988) and hepatopancreas (Tanchotikul and Hsieh., 1989), boiled or pasteurized crab meat (Matiella and Hsieh, 1990) and several dried and smoked fish products (Sakakibara et al., 1990).

4) Aromatic Hydrocarbons

Twenty-eight aromatic hydrocarbons were identified in

crab waste, but dimethylphenol was not detected after crab waste was treated with crawfish enzyme. Most aromatic compounds decreased due to enzyme treatment; however, the amount of toluene and phenol increased slightly and the content of benzene was not changed by enzyme treatment. This raises the possibility that crawfish enzymes can degrade higher molecular weight, potentially hazardous compounds to lower molecular weight compounds, i.e., benzene or toluene which are more easily removed from food due to their lower boiling point. The identity and function of crawfish enzymes responsible for degradation of alkylbenzenes is uncertain.

Eleven, thirteen and twenty alkylbenzenes were identified among the volatiles in the boiled crayfish tail meat (Vejaphan et al., 1988), hepatopancreas (Tanchotikul and Hsieh, 1989) and pasteurized crab meat (Matiella and Hsieh, 1990), respectively. The origin of alkylbenzene is uncertain but might originate from the environment (Habu et al., 1985). However, various alkylbenzene can be formed from beef fats during heating (Watanabe and Sato, 1971) and have been found in tea (Habu et al., 1985), corn (Buttery et al., 1978), nuts (Walradt et al., 1971; Crain and Tang, 1975), and dried and smoked Japanese fish products (Sakakibara et al., 1990).

5) Nitrogen-containing Compounds

The pyrazine compounds in the enzyme-treated crab waste increased approximately 1.2 to 4.6 times compared with

untreated waste. 2,5-Dimethylpyrazine was most abundant, followed by trimethylpyrazine among 12 pyrazine compounds detected in enzyme-treated crab waste. Untreated crab waste contained primarily 2,5-dimethylpyrazine followed by methylpyrazine. Two pyrazines, 2,5-dimethylpyrazine and trimethylpyrazine were reported as major components in crawfish waste (Tanchotikul and Hsieh, 1989; Baek et al., 1991; Cha et al., 1991). Among 12 pyrazine compounds, 2,3-dimethylpyrazine, 2-ethyl-6-methylpyrazine, tetramethylpyrazine, ethylpyrazine and 2,6-dimethylpyrazine contents in enzyme-treated crab waste were 4.6, 4.2, 3.6, 3.2, and 3.0 times higher, respectively, compared to untreated crab waste. This suggests that more precursors were produced by enzymatic reaction, resulting in the formation of higher concentrations of pyrazines.

Pyrazine compounds, which form through Maillard reactions, are generally considered as major volatile components in many thermal processed foods because they contribute to nutty or roasted aromas (Shibamoto, 1989). Pyrazine compounds have been reported as important volatile components in cooked krill and roasted shrimp (Kubota et al., 1980; 1986), crayfish tail meat (Vejaphan et al., 1988), crawfish hepatopancreas (Hsieh et al., 1989), and crawfish waste (Tanchotikul and Hsieh, 1989). The formation mechanism of alkylpyrazine compounds in the model system of the Maillard reaction has been investigated with regard to factors such as

temperature, heating time, reactant ratio, water activity, and pH effect (Koehler et al., 1969; Koehler and Odell, 1970; Reineccius et al., 1972; Shibamoto and Bernhard, 1976, 1977; Shibamoto et al., 1979).

2-Methylpyrazine, 2,5-dimethylpyrazine, 2-ethyl-5-methylpyrazine, 2,3,5-trimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were identified in volatiles from thermal degradation products of propyl-L-cysteine and glucose (Kimura et al., 1990). Baek et al. (1991) reported that the total amount of pyrazines in crayfish flavor concentrates formed at 100°C was 4.3-fold greater than at 85°C. The increased pyrazine compounds in enzyme treated crab waste might have resulted from higher level of amino and carbonyl groups produced by the action of hydrolytic enzymes in crawfish hepatopancreas. The present experiment suggests that more pyrazines can be produced by enzyme treatment prior to heating than by heat treatment alone.

Two pyridines were identified in both samples. 5-Ethyl-2-methylpyridine increased with enzyme treatment, whereas pyridine was not changed. 5-Ethyl-2-methylpyridine can be formed by the reaction of aldehydes with ammonia or amino groups (Noller, 1957).

Pyrrole, whose characteristics have been described as sweet, warm-ethereal, slightly burnt, and nauseating (Arctander, 1969), increased about 1.5 times due to enzyme treatment. However, pyrrole was not detected in the dynamic

headspace of boiled crab meat (Matiella and Hsieh, 1990). Although it may not contribute as much nutty flavor as alkylpyrazine, pyrrole could still be an important volatile component of crab waste in view of its higher peak area. The formation of pyrrole is possibly induced from proline and hydroxyproline in Strecker degradation (Heath and Reineccius, 1986). Proline or hydroxyproline can be produced by degradation of collagen in the crab waste by collagenolytic activity of crawfish enzyme. Thus, a higher content of pyrrole and methylpyrrole could be due to the collagenolytic activity of crawfish enzyme. Methylpyrrole, which has undesirable aroma at higher concentration, but sweet and burnt aroma when highly diluted (Shigematsu et al., 1972), increased 2.2-fold by enzyme treatment.

6) Sulfur-containing Compounds

Seven sulfur-containing compounds were identified in both treated and untreated crab waste. 3,5-Dimethyl-1,2,4-trithiolanes increased 3.6 and 3.7 fold, respectively, in the enzyme-treated crab waste. 3,5-Dimethyl-1,2,4-trithiolane isomers (cis and trans forms) produced onion-like aromas in boiled antarctic krills (Kubota et al., 1980). These compounds can be produced by the reaction of 2,4-decadienal and sulfur-containing amino acids (Ho et al., 1989). As shown in Table IV-15, 2,4-decadienal decreased considerably, which may be due to consumption as precursor to these sulfur containing

compounds. Also, dimethyl disulfide increased with enzyme treatment. However, dipropyl disulfide was considerably lower in enzyme treated crab waste. The reason is not clear as there was no change in other sulfur-containing compounds. Dimethyl disulfide, which has sulfurous odors reminiscent of cooked cabbage, was believed to be produced from decomposition of methionine (Deck et al., 1973).

Generally, sulfur-containing compounds play an important role in generating meaty aromas in a variety of meat products (Shahidi et al., 1986). A model system reaction of cysteine with thiamine, glutamate, and ascorbic acid produced a complex mixture of compounds with an overall flavor resembling that of roasted meat (Hartman et al., 1984a,b; Werkhoff et al., 1990). Large numbers of sulfur-containing compounds were distributed in cooked and fermented shrimp (Choi et al., 1983). These compounds may be important contributors to the odor of cooked krill, which has rancid, nutty aromas (Kubota et al., 1980).

The compound 2-thiophenecarboxaldehyde, having a raw green smell (Jayalekahmy and Narayanan, 1989), was not changed by the enzyme treatment. This compound was reported in roasted coffee (Vitzthym and Werkhoff, 1978) and white bread crust (Folkes and Gramshaw, 1977).

7) Miscellaneous Compounds

Sixteen miscellaneous flavor components were identified in both samples. 2-Methylfuran, 2-ethylfuran, pentylfuran and

octylfuran increased by 2.5, 1.6, 2.2, and 2.0 times, respectively, by enzyme treatment. Five saturated aliphatic hydrocarbons, n-octane, undecane, dodecane, pentadecane and heptadecane, were also higher in the enzyme treated crab waste, however, one unsaturated hydrocarbon, 2-octene decreased with enzyme treatment.

Hydrocarbons are minor but universal components of marine plants and animals. They may be biosynthesized, for instance by algae, and may also be a contaminate from petroleum (Sasaki et al., 1991). However, alkanes (C8-C19) which come from decarboxylation and splitting of carbon-carbon chains of higher fatty acids (Watanabe and Sato, 1971), are not significant flavor components in foods (Grosch, 1982; Flath et al., 1982). High levels of ethylacetate were detected in both samples but the enzyme treated sample decreased by 61%. Limonene was identified in both samples and its content was not changed by enzyme treatment. Limonene possibly ingested from algae, has been found as a volatile component in beef (Shahidi et al., 1986), fish (Josephson et al., 1984), krill (Kubota et al., 1982), shrimp (Kubota et al., 1986), crayfish (Vejaphan et al., 1989), and clam (Tanchotikul and Hsieh, 1991).

CHAPTER V

SUMMARY AND CONCLUSIONS

It has been found that crawfish develop a "mushy" texture during cold storage, which is an occasional and unpredictable problem. This textural defect is presumed to be due to degradation of muscle tissue associated with the hepatopancreas, as this phenomenon is most prominent in the anterior section of the tail meat. Trypsin is quantitatively important in the digestive system. Moreover, participation of other proteases in muscle degradation is affected by the presence of trypsin due to its unique activation function of other enzymes. To understand the effect of intestinal trypsin on muscle degradation in crawfish, it is necessary to establish isolation procedures and to characterize the enzymes.

The application of an enzyme for a particular food processing operation is influenced by several factors such as pH, temperature, cost, and specificity. Therefore, knowledge and understanding of these factors are essential in screening for alternative sources of enzymes for use as food processing aids.

Four electrophoretically homogenous trypsins designated trypsin A, B, C, and D were isolated from crawfish hepatopancreas through acetone fractionation and benzamidine Sepharose 6B affinity, DEAE-Sephacel, and Sephacryl S-200

gel chromatography. Purity was increased 56, 100, 87, and 64-fold with approximately 2.4, 8.1, 5.1, and 3.0% yield for trypsin A, B, C, and D, respectively. The molecular weights of trypsin A, B, C and D were estimated to be 23,800, 27,900, 24,800, and 31,400 D, respectively, using Sephacryl S-200 gel filtration. Isoelectric points of the enzymes were estimated to be approximately 3.0. The amino acid profiles of the trypsins revealed high levels of acidic and low level of basic amino acid residues, which is similar to other anionic trypsins from marine animals.

Purified enzymes had esterolytic activity for N-p-tosyl-L-arginine methyl ester (TAME) and benzoyl-L-arginine ethyl ester (BAEE), amidolytic activity for benzoyl-D,L-arginine-p-nitroanilide (BAPNA) and proteolytic activity for casein but lacked chymotryptic activity. Caseinolytic activity was consistently highest between pH 5.5 and 10.0 for all proteases. Amidolytic and esterolytic activities were maximum in a narrower range between pH 7.5 and 8.5. Temperature optima for hydrolysis of casein was 45°C for trypsin A and B, and 50°C for trypsin C and D. Temperature optima for hydrolysis of BAPNA at pH 6.8 was 60°C for trypsin A and B, and 65°C for trypsin C and D. The optimum pH for esterolytic activity toward TAME was pH 7.5 for trypsin A, C, and D and pH 8.0 for trypsin B. All trypsins had maximal activity at 60°C and pH 6.8, but maximal activity of trypsin A and B shifted to 55°C at pH 8.1 for

hydrolysis of TAME.

Thermal stabilities of enzymes increased in the presence of casein. Trypsin C and D had higher thermal stability, which may implicate them in the development of mushiness in blanched crawfish tail meat during storage at refrigeration temperature. Trypsins were unstable at acidic pH but stable at neutral and alkaline pH. The activities of trypsins increased in the presence of Ca^{2+} ions, and maximum activities of trypsin A, B, C, and D were achieved at concentrations of 0.5, 0.05, 0.025, and 0.1 mM Ca^{2+} ions, respectively.

Trypsins were highly sensitive to serine enzyme inhibitors i.e., diisopropyl fluorophosphate (DFP), phenyl-methylsulfonylfluoride (PMSF) and soybean trypsin inhibitor, and trypsin inhibitors such as N-p-tosyl-L-lysine chloromethyl ketone (TLCK), benzamidine, leupeptin, and antipain. Thus, these enzymes were shown to be trypsin-like serine enzymes of the type found in marine animals based on molecular weight, isoelectric point, amino acid composition, substrate specificity, and inhibition studies.

All enzymes, except trypsin B had similar K_m' value and V_{max} at pH 6.8 and 25°C for esterase reaction (TAME) and had lower K_m' values at pH 8.1 compared with pH 6.8. The K_m' values and V_{max} of crawfish trypsins were higher than those of bovine trypsin at pH 6.8. The K_m' values for the hydrolysis of BAPNA at pH 8.1 were much lower than at pH

6.8. The trypsins had different catalytic effects for amidolytic reaction at pH 6.8 and 8.1 on the basis of substrate turnover number. The K_m' values of crawfish trypsins for BAPNA hydrolysis were very low compared with that of bovine trypsin at pH 6.8 and 8.1. Trypsin C was the best catalyst for the amidolytic reaction at pH 8.1. K_m' values for the hydrolysis of casein by crawfish trypsin and bovine trypsin were similar at pH 6.8 and 8.1, but at pH 6.8 trypsin B had highest affinity toward casein. Trypsin D had the highest substrate turnover number for caseinolytic reaction followed by trypsin A compared with the other trypsins at pH 6.8. This suggests that these two enzymes may play a primary role in the development of mushiness in crawfish tail meat compared with other trypsins.

Crawfish trypsins had similar activation energies for the esterolytic reaction using TAME or BAEE of approximately 6.4 to 9.0 Kcal/mole. The activation energies for the hydrolysis of BAPNA ranged from 5.8 to 6.2 Kcal/mole and were considerably lower than for the esterolytic reaction. However, the activation energies for hydrolysis of casein varied among enzymes between 9.8 to 13.0 Kcal/mole, which are higher than those for esterolytic and amidolytic reaction.

Maximal hydrolysis of crab waste protein by a crude extract of crawfish enzyme was at pH 9.0 with 4 hr reaction time at 45°C. One-hundred fifteen volatile flavor components

were identified from enzyme-treated crab waste and one-hundred sixteen in crab waste. Higher peak areas were obtained for enzyme-treated crab waste than for untreated crab waste except for alkylbenzene, octenal, heptenal, and all dienals. Among 12 identified pyrazine compounds, 2,3-dimethylpyrazine, 2-ethyl-6-methylpyrazine, tetramethylpyrazine, ethylpyrazine, and 2,6-dimethylpyrazine contents in enzyme treated crab waste were 4.6, 4.2, 3.6, 3.2, and 3.0 times higher, respectively, compared with crab waste. Also, 3,5-Dimethyl 1,2,4-trithiolane increased considerably in enzyme treated crab waste.

In conclusion, four trypsins were isolated from crawfish hepatopancreas, which are presumed to be at least partially responsible for the "mushy" texture in crawfish tail meat noted during refrigerated storage. Having developed the purification scheme presented in this research, it now will be possible to further evaluate the effect of different processing conditions on enzyme activity. Furthermore, inferences made from the present research will aid in establishing optimal processing procedures to maximize storageability of processed crawfish products.

In addition to the aforementioned, the potential of the enzymes to hydrolyze different substrates, possibly with ultimate commercial significance, can be evaluated. An analysis of the feasibility of utilizing crawfish

hepatopancreas enzymes as an extractant for flavor development and protein products of hydrolysates from seafood waste or in other industrial processes may provide economic benefits to the crawfish processing industry.

Further studies are warranted to characterize the enzymes and to utilize seafood waste. These include:

- 1) Statistical determination of enzyme kinetic and thermodynamic parameters.
- 2) Characterization of crawfish lipolytic and amylolytic activities and their function in development of flavor and mushiness.
- 3) Establishment of minimal processing conditions to increase crawfish quality.
- 4) Development of economical large-scale enzyme isolation methods from seafood waste, i.e., fish intestine, for industrial utilization.
- 5) Optimization of enzymatic reaction conditions for production of a flavor base from crab or crawfish waste.
- 6) Quantification of increased volatile flavor compounds produced by enzyme reaction.
- 7) Comparison of flavor development initiated by fish enzymes from different sources.

LITERATURE CITED

- Ahern, T.J. and Klibanov, A.M. 1985. The mechanism of irreversible enzyme inactivation at 100°C. *Science* 228: 1280.
- Aksnes, A. and Brekken, B. 1988. Tissue degradation, amino acid liberation and bacterial decomposition of bulk stored capelin. *J. Sci. Food Agric.* 45: 53.
- Andrews, P. 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.* 91: 222.
- Anonymous. 1988. *Worthington Enzyme Manual*. C.C. Worthington, (Ed.), p. 93. Worthington Biochemical Co. Freehold, NJ.
- Anson, M.L. 1939. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin, *J. Gen. Physiol.* 22: 79.
- A.O.A.C. 1980. "Official Methods of Analysis." 13th Ed. Association of Official Analytical Chemists, Washington, DC.
- Arctander, S. 1969. "Perfume and Flavor Chemicals: Aroma chemicals," Vol. 1-2. S. Arctander, Publisher. NJ.
- Argos, P., Rossmann, M.G., Grau, U.M., Zuber, H., Frank, G. and Tratschin, J.D. 1979. Thermal stability and protein structure. *Biochem.* 18: 5698.
- Armstrong, J.R. and DeVillez, E.J. 1978. Proteases of low molecular weight in the digestive fluids of decapods. *Can. J. Zool.* 56: 2225.
- Arunchalam, K. and Haard, N.F. 1985. Isolation and characterization of pepsin from polar cod (*Boreogadus saida*). *Comp. Biochem. Physiol.* 80B: 467.
- Asgreisson, B., Fox, J.W. and Bjarnason, J.B. 1989. Purification and characterization of trypsin from the poikilotherm *Gadus morhua*. *Eur. J. Biochem.* 180: 85.
- Auld, D.S. 1988. Metal-free dialysis tubing. In "Methods in Enzymology". Vol. 158. J.F. Riordan and B.L. Vallee (Ed.). p. 13. Academic Press. NY.
- Baek, H.H., Cha, Y.J. and Hsieh, T.C.-Y. 1991. Effect of temperature on levels of pyrazines in crayfish flavor concentrates (In preparation).
- Bailey, M. E. and Einig, R. G. 1989. Reaction flavors of meat. In "Thermal Generation of Aromas" T.H. Parliment, R.J. McGorrin and C.T. Ho (Ed.). ACS Symposium Series 409, p. 421.

American Chemical Society, Washington, DC.

Baranowski, E.S., Nip, W.K. and Moy, J.H. 1984. Partial characterization of a crude enzyme extract from the freshwater prawn Macrobrachium rosenbergii. J. Food Sci. 49: 1494.

Barrett, A.J. 1972. Lysosomal enzymes. In "Lysozymes-A Laboratory Handbook," J.T. Dingle (Ed.). p. 46. North-Holland Publishing Co., NY.

Bewley, G.C. and Devillez, E.J. 1968. Isolation and characterization of the digestive proteinase in the earthworm Lumbricus terrestris Linneaus. Comp. Biochem. Physiol. 25: 1061.

Bio-rad. 1988. Protein Assay. Bio-rad. CA.

Bird, J.W.C., Schwartz, W.N. and Spanier, A.M. 1977. Degradation of myofibrillar proteins by cathepsins B and D. Acta Biol. Med. Germ. 36: 1587-1604.

Bradshaw, R.A., Neurath, H., Tye, R.W., Walsh, K.A. and Winter, W.P. 1970. Comparison of the partial amino-acid sequence of dogfish trypsinogen with bovine trypsinogen. Nature 226: 237.

Bricteux-Gregoire, S., Schyns, R. and Florkin, M. 1974. N-Terminal amino acid sequence of trypsinogen from the elephant seal Mirounga leonina I (Carnivora). Biochim. Biophys. Acta. 351: 87.

Bricteux-Gregoire, S., Schyns, R., Florkin, M., Emmens, Welling, G.W. and Beintema, J.J. 1975. N-Terminal amino acid sequence of trypsinogen from the lesser rorqual Balaenoptera acutorostrata (Cetacea). Biochim. Biophys. Acta, 386: 244.

Brockhoff, H., Hoyle, R.J. and Hwang, P.C. 1970. Digestive enzymes of the American lobster, Homarus americanus. J. Fish. Res. Bd. Can. 27: 1357.

Brodrick, J.W., Largman, C., Hsiang, M.W., Johnson, J.H. and Geokas, M.C. 1978. Human cationic trypsinogen. Purification, characterization, and characteristics of autoactivation. J. Biol. Chem. 253: 2732.

Brown, W.E. and Wold, F. 1973. Alkyl isocyanates as active-site-specific reagents for serine proteases: Identification of the active-site serine as the site of reaction. Biochem. 12: 835.

Brun, G.L. and Wojtowicz, M.B. 1976. A comparative study of the digestive enzymes in the hepatopancreas of jonah crab

(Cancer borealis) and rock crab (Cancer irroratus). Comp. Biochem. Physiol. 53B: 387.

Bundy, H.F. and Gustafson, 1973. Purification and comparative biochemistry of a protease from the starfish Pisaster giganteus. Comp. Biochem. Physiol. 44B: 241.

Burnette, J.A., Flick, G.J., Miles, J.R., Cry, R.L., St. Angelo, A.J. and Dupuy, H.P. 1983. Characterization and utilization of ocean quahog (Arctica islandica) clam juice as a liquid and dehydrated flavoring agent. J. Food Sci. 48: 353.

Busconi, L., Folco, E.J.E., Martone, C.B., Trucco, R.E. and Sanchez, J.J. 1989. Fish muscle cytoskeletal network: Its spatial organization and its degradation by an endogenous serine proteinase. Arch. Biochem. Biophys. 268: 203-2-8.

Buttery, R.G., Ling, L.C., Teranishi, R. and Mon, T.R. 1978. Roasted lamb fat: basic volatile components. J. Agric. Food Chem. 25: 1227.

Cha, Y.J., Baek, H.H. and Hsieh, T.C.-Y. 1991. Volatile components in flavor concentrates from crayfish waste (In preparation).

Camacho, Z., Brown, J.R. and Kitto, G.B. 1970. Purification and properties of trypsin-like protease from the starfish Dermasterias imbricata. J. Biol. Chem. 245: 3964.

Chapelle, S. 1978. The influence of acclimation temperature on the fatty acid composition of an aquatic crustacean (Carcinus maenas). J. Exp. Zool. 204: 337.

Chen, C.S., Gan, S.W. and Yan, T.R. 1978a. Studies on the protease on Antarctic krill J. Chin. Biochem. Soc. 7: 78.

Chen, C.S., Yan, T.R. and Chen, H.Y. 1978b. Purification and properties of trypsin-like enzymes and a carboxypeptidase A from Euphausia superba. J. Food Biochem. 2: 349.

Chen, H.M. and Meyers, S.P. 1982a. Extraction of astaxanthin pigment from crawfish waste using a soy oil extract. J. Food Sci. 47: 892.

Chen, H.M. and Meyers, S.P. 1982b. Effect of antioxidants on stability of astaxanthin pigment in crawfish waste and oil extract. J. Agric. Food Chem. 30: 469.

Chen, T. and Reisler, E. 1984. Tryptic digestion of rabbit skeletal myofibrils: An enzymatic probe of myosin cross-bridges. Biochem. 23: 2400.

- Chen, C.C., Liu, S.E., Wu, C.M. and Ho, C.T. 1986. Enzymic formation of volatile compounds in Shiitake mushroom (Lentinus edodes Sing.). In "Biogenesis of Aromas," T.H. Parliment and R. Croteau (Ed.). ACS Symposium Series 317, American Chemical Society, Washington, DC.
- Chen, T., Liu, J. and Reisler, E. 1987. Proteolysis and binding of myosin subfragment 1 to actin. J. Mol. Biol. 194: 565.
- Chen, C.S., Tsao, C.Y. and Jiang, S.T. 1989. Purification and characterization of proteases from the viscera of milkfish (Chanos chanos). J. Food Biochem. 12: 269.
- Cho, D.M., Pyeun, J.H., Byun, D.S. and Kim, C.Y. 1983. Proteolytic activity of the crude enzyme extracted from the digestive tract of marine gastropods. Bull. Korea. Fish. Soc. 16: 384.
- Choi, S.H., Kobayashi, A. and Yamanishi, T. 1983. Odor of cooked small shrimp, Acetes japonica Kishinouye: Difference between raw material and fermented product. Agric. Biol. Chem. 47: 337.
- Cohen, T., Gertler, A. and Birk, Y. 1981a. Pancreatic proteolytic enzymes from carp Cyprinus carpio-I. Purification and physical properties of trypsin, chymotrypsin, elastase and carboxypeptidase B. Comp. Biochem. Physiol. 69B: 639.
- Cohen, T., Gertler, A. and Birk, Y. 1981b. Pancreatic proteolytic enzymes from carp Cyprinus carpio-II. Kinetic properties and inhibition studies of trypsin, chymotrypsin and elastase. Comp. Biochem. Physiol. 69B: 647.
- Crain, Jr., W.O. and Tang, C.S. 1975. Volatile components of roasted macadamia nuts. J. Food Sci. 40: 207.
- Creighton, T.E. 1983. Proteins in solution. In "Proteins. Structure and Molecular Properties," T.E. Creighton (Ed.), p. 265. Freeman, NY.
- Davis, B.J. 1964. Disc-electrophoresis-II, method and application to human serum protein. Ann. New York Acad. Sci. 121: 404.
- Deck, R.E., Pokorny, J. and Chang, S.S. 1973. Isolation and identification of volatile compounds from potato chips. J. Food Sci. 38: 345.
- de Haen, C., Walsh, K.A. and Neurath, H. 1977. Isolation and amino-terminal sequence analysis of a new pancreatic trypsinogen of the African lungfish Protopterus aethiopicus.

Biochem. 16: 4421.

Dehlquist, F.W., Long, J.W. and Bigbee, W.L. 1976. Role of calcium in the thermal stability of thermolysin. Biochem. 15: 1103.

Dendinger, J.E. 1987. Digestive proteases in the midgut gland of the Atlantic blue crab, Callinectes sapidus. Comp. Biochem. Physiol. 88B: 503.

Dendinger, J.E. and O'Connor, K.L. 1990. Purification and characterization of a trypsin-like enzyme from the midgut gland of the atlantic blue crab, Callinectes sapidus. Comp. Biochem. Physiol. 95B: 525.

Depaola, A., Perkins, B.E., Harper, K.P. and McPhearson, R.M. 1989. Recovery of protein and microorganisms from shrimp peeler effluent. J. Food Sci. 54: 1660.

Desnuelle, P. 1960. Trypsin. In "The Enzymes". P.D. Boyer, H. Lardy and K. Myrback (Ed.). 2nd Ed. Vol. 4. p. 119, Academic Press. NY.

DeVillez, E.J. and Lau, J. 1970. Specificity and digestive function of alkaline proteinase from the gastric juice of the crayfish Orconectes virilis. Int. J. Biochem. 1: 108.

DeVillez, E.J. 1975. Current status concerning the properties of crustacean digestive proteinases. In "Freshwater crayfish". J.W. Avault, Jr. (Ed.), p. 195. Division of Continuing Education, Louisiana State Univ. Baton Rouge, LA.

Dziezak, J.D. 1991. Enzymes: Catalysts for food processes. Food Technol. 45(1): 77.

Eisen, A.Z. and Jeffery, J.J. 1969. An extractable collagenase from crustacean hepatopancreas. Biochim. Biophys. Acta, 191: 517.

Eisen, A.Z., Henderson, K.O., Jeffrey, J.J. and Bradshaw, R.A. 1973. A collagenolytic protease from the hepatopancreas of the fiddler crab, Uca pugnator: Purification and properties. Biochem. 12: 1814.

Erlanger, B.F., Kokowsky, N. and Cohen, W. 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95: 271.

Fahrney, D.E. and Gold, A.M. 1963. Sulfonyl fluorides as inhibitors of esterases. I. Rates of reaction with acetylcholinesterase, α -chymotrypsin, and trypsin. J. Am. Chem. Soc. 85: 997.

Figarella, C., Negri, G.A. and Guy, O. 1975. The two human trypsinogens. Inhibition spectra of the two human trypsins derives from their purified zymogens. Eur. J. Biochem. 53: 457.

Flath, R.A., Sugisawa, H. and Teranishi, R. 1982. Problem in flavor research. In "Flavor Research. Recent Advances", R. Teranishi, R.A. Flath, and H. Sugisawa, (Ed.), Marcel Dekker, Inc., NY.

Folin, O. and Ciocalteus, V. 1927. On tyrosine and tryptophan determination in proteins, J. Biol. Chem. 73: 627.

Galgani, F.F., Benjamin, Y. and Ceccaldi, H.J. 1984. Identification of digestive proteinases of Penaeus kerathrus (Forsk.) : A comparison with Penaeus japonicus bate. Comp. Biochem. Physiol. 78B: 355.

Galgani, F. and Nagayama, F. 1986. Characteristics of digestive proteolysis of the crabs Portunus trituberculatus, Portunus sanguinolentus and Charybdis japonica. Bull. Jap. Soc. Sci. Fish. 52: 2183.

Galgani, F. and Nagayama, F. 1987a. Digestive proteinases in five species of Lithodidae (Crustacea, Decapoda). Comp. Biochem. Physiol. 87B: 103.

Galgani, F. and Nagayama, F. 1987b. Digestive proteinases in the Japanese lobster Panulirus japonicus. Comp. Biochem. Physiol. 87B: 889.

Galgani, F. and Nagayama, F. 1988. Digestive proteolysis and digestive proteinases in deep sea crabs Geryon affinis and Chionoecetes japonicus. Nippon Suisan Gakkaishi. 54: 983.

Galliard, T. and Phillips, D.R. 1976. The enzymic cleavage of linoleic acid to C9 carbonyl fragments in extracts of cucumber (Cucumis sativus) fruit and the possible role of lipxygenase. Biochim. Biophys. Acta. 431: 278.

Gates, B.J. and Travis, J. 1969. Isolation and comparative properties of shrimp trypsin. Biochem. 8: 4483.

Geiger, R. and Fritz, H. 1981. Trypsin. In "Methods of Enzymatic Analysis". H.U. Bergmeyer (Ed.), 3rd Ed. Vol. IV, p. 119. Verlag Chemie. Florida.

Gildberg, A. 1978. Proteolytic activity and the frequency of burst bellies in capelin. J. Food Technol. 13: 409.

Gildberg, A. and Raa, J. 1983. Purification and characterization of pepsins from the arctic fish capelin

(Mallotus villosus). Comp. Biochem. Physiol. 75A: 337.

Godber, J.S., Wang, J., Moertle, G.M., Omoike, I.I., Finerty, M.W., Grodner, R.M. and Moody, M.W. 1986. The effect of residual hepatopancreatic tissue and freezing method on proteolytic activity in crayfish tail meat as related by free amino acids and texture. Abstract No. 394, 47th Annual Meeting of the Institute of Food Technologists, Dallas, Tx.

Grant, G.A. and Eisen, A.Z. 1980. Substrate specificity of the collagenolytic serine protease from the fiddler crab Uca pugilator. Studies with noncollagenous substrates. Biochem. 19: 6089.

Grant, G.A., Sacchettini, J.C. and Welgus, H.G. 1983. A collagenolytic serine protease with trypsin-like specificity from the fiddler crab Uca pugilator. Biochem. 22: 354.

Greenberg, M.J. 1981. Characterization of poultry byproduct meat flavor volatiles. J. Agric. Food Chem. 29: 831.

Grosch, W. 1982. Lipid degradation products and flavor. In "Developments in Food Science 3A. Food Flavors. Part A. Introduction," I.D. Morton, and A.J. MacLeod (Ed.), Elsevier Sci. Publishing Co., Inc., New York.

Guizani, N., Rolle, R.S., Marshall, M.R. and Wei, C.I. 1991. Isolation, purification and characterization of a trypsin from the pyloric caeca of mullet (Mugil cephalus). Comp. Biochem. Physiol. 98B: 517.

Habu, T., Klath, R.A., Mon, R.A. and Morton, J.F., 1985. Volatile components of rooibos tea (Aspalathus linearis). J. Agric. Food Chem. 33: 249.

Hang, Y.D., Woodams, E.E. and Parsons, G.F. 1980. Isolation and chemical evaluation of protein from clam wash water. J. Food Sci. 45: 1040.

Hara, K., Arano, H. and Ishihara, T. 1984. Some enzymatic properties of alkaline proteinases from the rotifer, Brachionus phicatilis. Bull. Jap. Soc. Sci. Fish. 50: 1611.

Hartman, G.J., Scheide, J.D. and Ho, C.T. 1984a. Effect of water activity on the major volatiles produced in a model system approximating cooked meat. J. Food Sci. 49: 607.

Hartman, G.J., Scheide, J.D. and Ho, C.T. 1984b. Volatile products formed from a flavor model system at high and low moisture levels. Lebensm.-Wiss. Technol. 17(4): 222.

Heath, H.B. and Reineccius, G. 1986. Flavor and its study. In

"Flavor Chemistry and Technology." AVI Publishing Co. Inc. Westport, CT.

Heu, M.S., Pyeun, J.H., Kim, H.R. and Godber, J.S. 1991. Purification and characterization of alkaline proteinases from the viscera of anchovy, Engraulis japonica. J. Food Biochem. 15: 51.

Hewlett Packard Co. 1988. Wiley/NBS Data base (PBM format).

Hjelmeland, K. and Raa, J. 1982. Characteristics of two trypsin type isozymes isolated from the arctic fish capelin (Mallotus villosus). Comp. Biochem. Physiol. 71B: 557.

Ho, C. T., Bruechert, L. J., Zhang, Y. and Chiu, E. M. 1989. Contribution of lipids to the formation of heterocyclic compounds in model systems. In "Thermal generation of aromas." T.H. Parliment, R.J. McGorrin and C.T. Ho (Ed.). ACS Symposium Series 409, p. 105. American Chemical Society, Washington, DC.

Honjo, I., Kimura, S. and Nonaka, M. 1990. Purification and characterization of trypsin-like enzyme from shrimp Penaeus indicus. Nippon Suisan Gakkaishi. 56: 1627.

Hood, L.F., Zall, R.R. and Conway, R.L. 1976. Conversion of minced clam wash water into clam juice: waste handling or product development. Food Prod. Develop. 10: 86.

Hsieh, T. C.-Y., Vejaphan, W., Williams, S. S. and Matiella, J. E., 1989. Volatile flavor components in thermally processed Louisiana red swamp crayfish and blue crab. In "Thermal Generation of Aromas." T.H. Parliment, R.J. McGorrin and C.T. Ho (Ed.). ACS Symposium Series 409, p. 386. American Chemical Society, Washington, DC.

Hummel, B.C. 1959. A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. Can. J. Physiol. 37: 1393.

Iida, Y., Nakagawa, T. and Nakayama, F. 1991. Properties of collagenolytic proteinase in Japanese spiny lobster and horsehair crab hepatopancreas. Comp. Biochem. Physiol. 98B: 403.

Iwata, K., Kobashi, K. and Hase, J. 1974. Studies on fish muscle alkaline proteinase-III. Distribution of alkaline proteinase in muscle of freshwater fish, marine fish and internal organs of carp. Bull. Jap. Soc. Sci. Fish. 40: 201.

Jany, K.D. 1976. Studies on the digestive enzymes of the stomachless bonefish Carassius auratus gibelio (bloch); Endopeptidases. Comp. Biochem. Physiol. 53B: 31.

Jaswal, A.S. 1990. Amino acid hydrolysate from crab processing waste. J. Food Sci. 55: 379.

Jayalekahmy, A. and Narayanan, C. S. 1989. Flavor constituents of roasted cashew nuts. In "Thermal Generation of Aromas" T.H. Parliment, R.J. McGorin and C.T. Ho (Ed.). ACS Symposium Series 409, p. 355. American Chemical Society, Washington, DC.

Jiang, S.T., Moody, M.W. and Chen, H.C. 1991. Purification and characterization of proteases from digestive tract of grass shrimp (Penaeus monodon). J. Food Sci. 56: 322.

Joh, Y and Hood, L.F. 1979. Preparation and properties of dehydrated clam flavor from clam processing wash water. J. Food Sci. 44: 1612.

Josephson, D.B., Lindsay, R.C. and Stuiber, D.A. 1983. Identification of compounds characterizing the aroma of whitefish (Coregonus clupeaformis). J. Agric. Food Chem. 31: 326.

Josephson, D.B., Lindsay, R.C. and Stuiber, D.A. 1984. Variations in the occurrences of enzymatically derived volatile aroma compounds in salt- and freshwater fish. J. Agric. Food chem. 32: 1344.

Josephson, D.B. and Lindsay, R.C. 1986. Enzymic generation of volatile aroma compounds from fresh fish. In "Biogenesis of Aromas," T.H. Parliment and R. Croteau, (Ed.). ACS Symposium Series 317, American Chemical Society, Washington DC.

Kafatos, F.C., Law, J.H. and Tartakoff, A.M. 1967. Cocconase-II. Substrate specificity, inhibitors and classification of enzyme. J. Biol. Chem. 242: 1488.

Kakade, M.L., Simons, N. and Liener, I.E. 1969. An evaluation of natural vs. synthetic substrates for measuring the antitryptic activity of soybean samples. Cereal Chem. 46: 518.

Kalac, J. 1978. Studies on herring (Clupea harengus L.) and capelin (Mallotus villosus L.) pyloric caeca proteases III: Characterization of the anionic fractions of chymotrypsins. Biologia. 33: 939.

Karahadian, C. and Lindsay, R. C. 1989. Flavor Chemistry: Trends and Developments. R. Teranishi, R.G. Buttery and F. Shahidi, (Ed.), p. 60. American Chemical Society, Washington, DC.

Keil, B. 1971. Trypsin. In "The Enzymes", Vol. iii, pp.249-275. P.D. Boyer, (Ed.), Academic Press, NY.

Keithly, Jr. W.R., Roberts, K.J. and Liebzeit, A.W. 1988. Louisiana Blue Crab Production, Processing, and Markets. Louisiana Sea Grant College Program, Louisiana State University, LA.

Kim, H. R. 1986. Purification and characterization of the three alkaline proteinases from the pyloric caeca of mackerel, Scomber japonicus. M.S thesis of Nat'l. Fish. Univ. of Pusan. Pusan.

Kim, H.R., Pyeun, J.H. and Cho, J.G. 1986. Proteolytic enzymes distributed in the tissues of dark fleshed fish. 2. Comparison of the proteolytic activity of the tissue extracts from the intestinal organs of mackerel and sardine. Bull. Korean Fish. Soc. 19: 521.

Kim, H.R. and Pyeun, J.H. 1986. The proteinase distributed in the intestinal organs of fish. 2. Characterization of the three alkaline proteinases from the pyloric caeca of mackerel Scomber japonicus. Bull. Korean Fish. Soc. 19: 547.

Kim, H.R., Godber, J.S. and Pyeun, J.H. 1989a. Evaluation of proteolytic enzymes from the hepatopancreas of crawfish (Procambarus clarkii). Proceedings in Tropical and Subtropical Seafood Technologists. 13: 97.

Kim, H.R., Godber, J.S. and Pyeun, J.H. 1989b. Purification and characterization of trypsin-like serine protease from crawfish hepatopancreas. 50th Annual Meeting of the Institute of Food Technologists, Chicago. IL.

Kim, H.R. and Godber, J.S. 1990. Substrate specificity of a trypsin-like serine protease isolated from crawfish hepatopancreas. 50th Annual Meeting of the Institute of Food Technologists, Anaheim. CA.

Kimoto, K., Thanh, V.V. and Murakami, K. 1981. Acid proteinases from Antarctic krill, Euphausia superba. Partial purification and some properties. J. Food Sci. 46: 1881.

Kimoto, K., Kusama, S. and Murakami, K. 1983. Purification and characterization of serine proteinases from Euphausia superba. Agric. Biol. Chem. 47: 529.

Kimoto, K., Fukamizu, A. and Murakami, K. 1986. Partial purification and characterization of proteinases from abdomen part muscle of Antarctic krill. Bull. Jap. Soc. Sci. Fish. 52: 745.

Kimura, K., Iwata, I., Nishimura, H., Kasuya, M., Yamane, A. and Mizutani, J. 1990. Thermal degradation products of S-alkyl-L-cysteine occurring in the Allium species with D-

glucose. Agric. Biol. Chem. 54: 1893.

Kinlin, T. E., Walradt, J. P. and Denton, W. 1974. In Freshwater Crayfish: International Symposium on Freshwater Crayfish, J.W. Avault, Jr. (Ed.), p. 175. Louisiana State Univ., Div Continuing Education, Baton Rouge, LA.

Koehler, P.E., Mason, M.E. and Newell, J.A. 1969. Formation of pyrazine compounds in sugar-amino acid model systems. J. Agric. Food Chem. 17: 393.

Koehler, P.E. and Odell, G.V. 1970. Factors affecting the formation of pyrazine compounds in sugar-amine reactions. J. Agric. Food Chem. 18: 895.

Komatsu, S.K. and Feeney, R.E. 1970. A heat labile fructose diphosphate aldolase from cold-adapted Antarctic fishes. Biochim. Biophys. Acta, 206: 305.

Konakaya, S. 1980. Protease activity and autolysis of Antarctic krill. Bull. Jap. Soc. Sci. Fish. 46: 175.

Konigsbacher, K.S. and Hewitt, E.J. 1964. Enzymatic odor development. Ann. New York Acad. Sci. 116: 705.

Kozlovskaya, E.P. and Elyakova, L.A. 1974. Purification and properties of trypsin-like enzymes from the starfish Lyastrosoma anthocticta. Biochem. Biophys. Acta. 371: 63.

Krieger, M., Kay, L.M. and Stroud, R.M. 1974. Structure and specific binding of trypsin: Comparison of inhibited derivatives and a model for substrate binding. J. Mol. Biol. 83: 209.

Krzeczowski, R.A., Tenney, R.D. and Kelley, C.E. 1971. Alaska king crab: Fatty acid composition, carotenoid index and proximate analysis. J. Food Sci. 36: 604.

Kubota, K., Kobayashi, A. and Yamanishi, T. 1980. Some sulfur containing compounds in cooked odor concentrate from boiled antarctic krill (Euphausia superba Dana). Agric. Biol. Chem. 44: 2677.

Kubota, K., Kobayashi, A. and Yamanishi, T. 1982. Basic and neutral compounds in the cooked odor from Antarctic krill. Agric. Biol. Chem. 46: 2835.

Kubota, K., Shijimaya, H. and Kovayashi, A. 1986. Volatile components of roasted shrimp. Agric. Biol. Chem. 50: 2867.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227: 680.

- Laskowski, M. 1955. Trypsinogen and trypsin. In "Methods in Enzymology". S.P. Colowick and N.O. Kaplan (Ed.), p. 26. Vol. II, Academic Press. NY.
- Lee, P.G., Blake, N.J. and Rodrick, G.E. 1980. A quantitative analysis of digestive enzymes for the freshwater prawn Macrobrachium rosenbergii. Proc. World Maricul. Soc. 11: 392.
- Lee, K.S. 1986. Evaluation of crawfish by-products in pigmentation of egg yolk. M.S. Thesis, Louisiana State University, Baton Rouge, LA.
- Lovell, S.J. and Harrington, W.F. 1981. Measurement of the fraction of myosin heads bound to actin in rabbit skeletal myofibrils in rigor. J. Mol. Biol. 149: 659.
- Liken, S. T. and Nickerson, G. B. 1964. Detection of certain hop oil constituents in brewing products. Am. Soc. Brew. Chem. Proc. 5: 13.
- Lindner, O., Angel, S., Weinberg, Z.R. and Granit, R. 1988. Study of factors inducing mushiness in stored prawns. Food Chem. 29: 119.
- Lindner, O., Angel, S., Weinberg, Z.R. and Granit, R. 1989. Study of the proteolytic activity of the hepatopancreas of the freshwater prawn, Macrobrachium rosenbergii, and its role in inducing mushiness in muscle tissue during post-mortem storage. Food Chem. 32: 19.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265.
- Lu, P.J., Liu, H.C. and Tsai, I.H. 1990. The midgut trypsins of shrimp (Penaeus monodon). High efficiency toward native protein substrates including collagens. Biol. Chem. Hoppe-Seyler. 371: 851-859.
- Magalhaes-Rocha, N.M., Rogana, E. and Mares-Guia, M. 1980. Kinetic parameters for the activation of α -trypsins by the methyl ester of tosyl-L-arginine (Tos-L-Arg-OMe). Arch. Biochem. Biophys. 200: 61.
- Makinodan, Y. and Hujita, M. 1990. Textural degradation of cooked fish meat gel (Kamaboko) by the addition of an edible mushroom, Judas' Ear (Auricularia auriculajudae (Fr.) Quel). J. Food Sci. 55: 979.
- Mandl, I., MacLennan, J.D., Howes, E.L., DeBellis, R.H. and Sohler, A. 1953. Isolation and characterization of proteinase and collagenase from Cl. histolyticum. J. Clin. Invest. 32:

1323.

Marshall, G.A., Moody, M.W., Hackney, C.R. and Godber, J.S. 1987. Effect of blanching time on the development of mushiness in ice-stored crawfish meat packed with adhering hepatopancreas. J. Food Sci. 52: 1504.

Martinez, A., Olsen, R.L. and Serra, J.L. 1988. Purification and characterization of two trypsin-like enzymes from the digestive tract of anchovy Engraulis encrasicolus. Comp. Biochem. Physiol. 91B: 677.

Matiella, J.E. and Hsieh, T.C.-Y. 1990. Analysis of crab meat volatile compounds. J. Food Sci. 55: 962.

McGill, A.S., Hardy, R., Burt, J.R. and Gunstone, F.D. 1977. Further analysis of the volatile components of frozen cold stored cod and the influence of these on flavour. J. Sci. Food Agric. 28: 200.

McLeese, J.M. and Stevens, E.D. 1982. The effect of acclimation temperature, assay temperature, and ration on the specific activity of trypsin and chymotrypsin from rainbow trout Salmo gairdneri. Comp. Biochem. Physiol. 73B: 631.

Meyers, S.P. and Chen, H.M. 1985. Process for the utilization of shellfish wastes. U.S. Patent 4,505,936.

Mornet, D., Bertrand, R., Pantel, P., Audemard, E. and Kassab, R. 1981. Proteolytic approach to structure and function of actin recognition site in myosin heads. Biochem. 20: 2110.

Mozhaev, V.V. and Martinek, K. 1984. Structure-stability relationships in proteins: new approaches to stabilizing enzymes. Enzyme Microb. Technol. 6: 50.

Murakami, K. and Noda, M. 1981. Studies on proteinases from the digestive organs of sardine. I. Purification and characterization of three alkaline proteinases from the pyloric caeca. Biochim. Biophys. Acta. 658: 17.

Muramatsu, T. and Morita, T. 1981. Anionic trypsin-like enzymes from the crab Eriocheir japonicus de Haan active in more acidic media. Comp. Biochem. Physiol. 70B: 527.

Nam, T.J. and Pyeun, J.H. 1983. Optimum condition of alkaline protease activity in muscle and internal organs of fish. Bull. Korean Fish. Soc. 16: 147.

Neito, M., Beltran, A. and Toledo, R.T. 1990. Extraction and characteristics of flavor concentrate from mechanically recovered crab meat. Proceedings in Tropical and Subtropical

Seafood Technologists. 14: 225.

Nip, W.K., Lan, C.Y. and Moy, J.H. 1985. Partial characterization of a collagenolytic enzyme fraction from the hepatopancreas of the freshwater prawn, Macrobrachium rosenbergii. J. Food Sci. 50: 1187.

No, H.K. and Meyers, S.P. 1989. Crawfish chitosan as a coagulant in recovery of organic compounds from seafood processing streams. J. Agric. Food Chem. 37: 580.

Noda, M. and Murakami, K. 1981. Studies on proteinase from the digestive organs of sardine. II. Purification and characterization of two acid proteinases from the stomach. Biochim. Biophys. Acta. 658: 27.

Noller, C. 1957. Chemistry of organic compounds. W.B. Saunders (Ed.). p. 627. Philadelphia.

Ohlsson, K. and Tegner, H. 1973. Anionic and cationic dog trypsin. Isolation and partial characterization. Biochim. Biophys. Acta. 312: 328.

Olafsdottir, S., Magnusson, S. and Bjarnason, J.B. 1985. Proteolytic degradation of muscle during the salt-curing process of herring. In "Aspartic Proteinases and Their Inhibitors". Walter de Gruyter & Co. Berlin. p. 561.

Olafson, R.W. and Smillie, L.B. 1975. Enzymatic and physicochemical properties of Streptomyces griseus trypsin. Biochem. 14: 1161.

Olsen, R.L., Johansen, A. and Myrnes, B. 1990. Recovery of enzymes from shrimp waste. Proc. Biochem. 25: 67.

Ooshiro, Z. 1968. Studies on proteinase in the pyloric caeca of fishes-I. Isolation and purification of proteinase from pyloric caeca of mackerel. Bull. Jap. Soc. Sci. Fish. 34: 847.

Ooshiro, Z. 1971. Studies on proteinase in the pyloric caeca of fishes-IV. Kinetic studies on the hydrolysis reaction of N_α-benzoyl-L-arginineamide catalyzed by mackerel proteinase. Bull. Jap. Soc. Sci. Fish. 37: 1110.

Osnes, K.K. and Mohr, V. 1985a. Peptide hydrolases of antarctic krill, Euphausia superba. Comp. Biochem. Physiol. 82B: 599.

Osnes, K.K. and Mohr, V. 1985b. On the purification and characterization of three anionic, serine-type peptide hydrolases from antarctic krill, Euphausia superba. Comp.

Biochem. Physiol. 82B: 607.

Osnes, K.K., Ellingsen, T.E. and Mohr, V. 1986. Hydrolysis of proteins by peptide hydrolases of antarctic krill, Euphausia superba. Comp. Biochem. Physiol. 83B: 801.

Osnes, K.K. and Mohr, V. 1986. On the purification and characterization of exopeptidases from antarctic krill, Euphausia superba. Comp. Biochem. Physiol. 83B: 445.

Overnell, J. 1973. Digestive enzymes of the pyloric caeca and of their associated mesentery in the cod (*Gadus morhus*). Comp. Biochem Physiol. 46B: 519.

Pakrashi, S.C., Dutta, P.K., Achari, B., Misra, S., Choudhury, A., Chattopadhyay, S. and Ghosh, A. 1989. Lipids and fatty acids of the horseshoe crabs Tachypleus gigas and Carcinoscorpius rotundicauda. Lipids 24: 443.

Perkins, B.E. and Meyers, S.P. 1977. Recovery and application of organic wastes from the Louisiana shrimp canning industry. Proceedings of the 8th National Symposium on Food Processing Wastes. p. 292. EPA-600/2-77-184, EPA. Washington DC.

Peterson, R.J. and Chang, S.S. 1982. Identification of volatile flavor compounds of fresh, frozen beef stew and a comparison of these with those of canned beef stew. J. Food Sci. 47: 1444.

Pfleiderer, G., Zwilling, R. and Sonneborn, H.H. 1967. Eine protease vom molekulargewicht 11,000 und eine trypsinähnliche Fraktion aus Astacus fluviatilis Fabr. Z. Physiol. Chem. 348: 1319.

Pivnenko, T.N., Epshtein, M.L., Kolodzeiskaya, M.V. and Kudinov, S.A. 1990. Purification and properties of trypsin from pyloric ceca of Pacific salmon. Appl. Biochem. Microbiol. 25: 412. (Prikl. Biokhim. Mikrobiol. 25; 490.)

Prahl, J.W. and Neurath, H. 1966. Pancreatic enzymes of the spiny Pacific dogfish. I. Cationic chymotrypsinogen and chymotrypsin. Biochem. 5: 2131.

Pyeun, J.H., Cho, D.M., Choi, Y.J., Byun, D.S. and Chung, T.H. 1983. Optimum condition of proteolytic activity of tissue extract from the digestive tract of marine mollusca. Publ. Inst. Mar. Nat. Fish. Uni. Pusan. 14: 59.

Pyeun, J.H., Kim, H.R. and Heu, M.S. 1988. The proteinase distributed in the intestinal organs of fish. 3. Purification and some enzymatic properties of the alkaline proteinases from the pyloric caeca of skipjack, Katsuwonus vagans Bull. Korean

Fish. Soc. 21: 85.

Pyeun, J.H., Kim, H.R. and Godber, J.S. 1989. Isolation and characterization of two trypsin-like enzymes from menhaden, Brevoortia tyrannus, intestine. Proceedings in Tropical and Subtropical Seafood Technologists. 14: 50.

Pyeun, J.H., Kim, H.R. and Godber, J.S. 1990. Comparative studies on the enzymatic properties of two trypsin-like enzymes from menhaden, Brevoortia tyrannus. Bull. Korean Fish. Soc. 23: 12.

Raae, A.J. and Wather, B.T. 1989. Purification and characterization of chymotrypsin, trypsin and elastase like proteinases from cod (Gadus morhua L.). Comp. Biochem. Physiol. 93B: 317.

Reddy, N.R., Flick, G.J., Dupuy, H.P. and Boardman, G.D. 1989. Characterization and utilization of dehydrated wash waters from clam processing plants as flavoring agents. J. Food Sci. 54: 55.

Reece, P. 1988. Recovery of proteases from seafood waste. Proc. Biochem. 23: 62.

Reeck, G.R., Winter, W.P. and Neurath, H. 1970. Pancreatic enzymes of the African lungfish Protopterus aethiopicus. Biochem. 9: 1398.

Reeck, G.R. and Neurath, H. 1972. Pancreatic trypsinogen from the African lungfish. Biochem. 11: 503.

Reineccius, G.A., Keeny, P.G. and Weissberger, W. 1972. Factors affecting the concentration of pyrazines in cocoa beans. J. Agric. Food Chem. 20: 202.

Rick, W. and Fritch, W.P. 1974. Pepsin. In "Methods of Enzymatic Analysis," H.U. Bergmeyer, (Ed.), p. 1052. Academic Press, NY.

Rowland, B., Finne, G. and Tillman, R. 1982. A morphological study of muscle proteolysis in the tail of Macrobrachium rosenbergii. Proceedings in 7th Annual Tropical and Subtropical Fisheries Technological Conference of the Americas, New Orleans, LA. p. 105.

Ryan, C.A. 1965. Chicken chymotrypsin and turkey trypsin. Arch. Biochem. Biophys. 110: 169.

Sakakibara, H., Ide, J., Yanai, T., Yajima, I. and Hayashi, K. 1990. Volatile flavor compounds of some kinds of dried and smoked fish. Agric. Biol. Chem. 54: 9.

Sasaki, S., Ota, T., Yamaguchi, H. and Takagi, T. 1991. Hydrocarbon components of salmons in the gulf of Alaska. *Nippon Suisan Gakkaishi*, 57: 111.

Schoellmann, G. and Shaw, E. 1963. Direct evidence for the presence of histidine in the active center of chymotrypsin. *Biochem* 2: 252.

Schroeder, D.D. and Shaw, E. 1968. Chromatography of trypsin and its derivatives. Characterization of a new active form of bovine trypsin. *J. Biol. Chem.* 243: 2943.

Schwert, G.W. and Takenaka, Y. 1955. A spectrophotometric determination of trypsin and chymotrypsin. *Biochim. Biophys. Acta.* 16: 570.

Seki, N., Sakaya, H. and Onozawa, T. 1977. Studies on proteases from Antarctic krill. *Bull. Jap. Soc. Sci. Fish.* 43: 955.

Severin, E.S. and Tomasek, V. 1965. Studies on active site of trypsin. I. The reaction of trypsin with specific inhibitor N α -tosyl-L-lysyl-chloromethane and certain pseudosubstrates 33. *Biochem. Biophys. Res. Comm.* 20: 496.

Shahidi, F., Rubin, L.J. and D'Souza, L.A. 1986. Meat flavor volatiles: a review of the composition, techniques of analysis, and sensory evaluation. *CRC Crit. Rev. Food Nutr.* 24: 141.

Shaw, E., Mares-Guia, M. and Cohen, W. 1965. Evidence for an active-center histidine in trypsin through use of a specific reagent, 1-chloro-3-tosylamido-7-amino-2-heptanone, the chromethyl ketone derived from N α -tosyl-L-lysine. *Biochem.* 4: 2219.

Shaw, E. 1975. Synthetic protease inhibitors acting by affinity labeling. In "Protease and Biological Control." E. Reich, D.B. Riffin and E. Shaw (Ed.). p. 455. Cols Spring Harbor Lab. Presss. NY.

Shiau, C.Y. and Chai, T. 1990. Characterization of oyster shucking liquid wastes and their utilization as oyster soup. *J. Food Sci.* 55(2): 34.

Shibamoto, T. and Bernhard, R. A. 1976. Effect of time, temperature, and reactant ratio on pyrazine formation in model systems. *J. Agric. Food Chem.* 24: 847.

Shibamoto, T. and Bernhard, R. A. 1977. Investigation of pyrazine formation pathways in sugar-ammonia model systems. *J.*

Agric. Food Chem. 25: 609.

Shibamoto, T., Akiyama, T., Sakaguchi, M., Enomoto, Y. and Masuda, H. 1979. A study of pyrazine formation. J. Agric. Food Chem. 27: 1027.

Shibamoto, T. 1989. Volatile flavor chemicals formed by the Maillard reaction. In "Thermal Generation of Aromas". T.H. Parliment, R.J. McGorin and C.T. Ho. (Ed.), p. 134. ACS Symposium Series 409, American Chemical Society, Washington, DC.

Shigematsu, H., Kurata, T., Kato, H. and Fujimaki, M. 1972. Volatile compounds formed on roasting D,L-alanine with D-glucose. Agric. Biol. Chem. 36: 1631.

Shin, D.H. and Zall, R.R. 1986. Purification and identification of a trypsin-like enzyme from the pyloric caeca of cod. Process Biochem. 19: 111.

Simpson, B.K. and Haard, N.F. 1984a. Trypsin from Greenland cod, Gadus ogac. Isolation and comparative properties. Comp. Biochem. Biophys. 79B: 613.

Simpson, B.K. and Haard, N.F. 1984b. Purification and characterization of trypsin from the Greenland cod, (Gadus ogac). I. Kinetic and thermodynamic characteristics. Can. J. Biochem. Cell Biol. 62: 894.

Simpson, B.K., Smith, J.P., Yaylayan, V. and Haard, N.F. 1989. Kinetic and thermodynamic characteristics of a digestive protease from Atlantic cod, Gadus morhua. J. Food Biochem. 13: 201.

Simpson, B.K., Simpson, M.V. and Haard, N.F. 1990. Properties of trypsin from the pyloric ceca of Atlantic cod (Gadus morhua). J. Food Sci. 55: 959.

Squires, E.J., Haard, N.F. and Feltham, L.A. 1986. Gastric proteases of the Greenland cod Gadus ogac. I. Isolation and kinetic properties. Biochem. Cell Biol. 64: 205.

Steel, R.G.D. and Torrie, J.H. 1980. "Principles and Procedures of Statistics," 2nd ed. McGraw-Hill, NY.

Stevens, E.D. and McLeese, J.M. 1988. Kinetics of trypsin from rainbow trout are not influenced by level of feeding. Comp. Biochem. Physiol. 90B: 375.

Stevenson, K.J. and Voordouw, J.K. 1975. Characterization and trypsin and elastase from the moose (Alces alces). I. Amino acid composition and specificity towards polypeptides.

Biochim. Biophys. Acta, 386: 324.

Stewart, K.K. 1973. A method for automated analyses of the activities of trypsin, chymotrypsin and their inhibitors. Anal. Biochem. 51: 11.

Stocker, W., Wolz, R.L. and Zwilling, R. 1988. Astacus protease, a zinc metalloenzyme. Biochem. 27: 5026.

Tanchotikul, U. and Hsieh, T. C.-Y. 1989. Volatile flavor components from crayfish waste. J. Food Sci. 54: 1515.

Tanchotikul, U. and Hsieh, T. C.-Y. 1991. Analysis of volatile flavor components in steamed rangia clam by dynamic headspace sampling and simultaneous distillation and extraction. J. Food Sci. 56: 327.

Tanford, C. 1968. Protein denaturation. Adv. Protein Chem. 23: 121.

Titani, K., Ericsson, L.H., Neurath, H. and Walsh, K.A. 1975. Amino acid sequence of dogfish trypsin. Biochem. 14: 1358.

Titani, K., Sasagawa, T., Woodbury, R.G., Ericsson, L.H., Dorsam, H. Kraemer, M., Neurath, H. and Zwilling, R. 1983. Amino acid sequence of crayfish (Astacus fluviatilis). Biochem. 22: 1459.

Torrissen, O., Tidemann, E., Hansen, F. and Raa, J. 1981. Ensiling in acid. A method to stabilize astaxanthin in shrimp processing by-products and improved uptake of this pigment by rainbow trout (Salmo gairdneri). Aquaculture, 26: 77.

Toyohara, H., Sakata, T., Yamashita, K., Kinoshita, M. and Shimizu, Y. 1990. Degradation of oval-filefish meat gel caused by myofibrillar proteinase(s). J. Food Sci. 55: 354.

Travis, J. and Roberts, R.C. 1969. Human trypsin, isolation and physical-chemical characterization. Biochem. 8: 2882.

Travis, J. 1968. Studies on the active site of sheep trypsin. Biochem. Biophys. Res. Commun. 30: 730.

Tressel, R., Bahri, D. and Engel, K.-H. 1981. In "Quality of Selected Fruits and Vegetables of North America", R. Teranishi and H. Barrera-Benitez (Ed.). p. 213, American chemical Society. Washington DC.

Uchida, N., Tsukayama, K. and Nishida, E. 1984. Purification and some properties of trypsins from pyloric caeca of chum salmon. Bull. Jap. Soc. Sci. Fish. 50: 129.

van den Dool, H. and Kratz, P. D. 1963. A generalization of the retention index system including linear temperature programmed gas liquid partition chromatography. J. Chromatogr. 11: 463.

van der Horst, D.J., Oudejans, R.C.H.M., Plug, A.G. and van der Sluis, I. 1973. Fatty acids of the female horseshoe crab Xiphosura (Limulus) polyphemus. Mar. Biol. 20: 291.

Vandermeers, A., Vandermeers-Piret, M.C., Rathe, J. and Christophe, J. 1973. Substrate activation of rat trypsins 1 and 2. Arch. Biochem. Biophys. 159: 492.

Vejaphan, W., Hsieh, T. C.-Y. and Williams, S. S. 1988. Volatile flavor components from boiled crayfish (Procambarus clarkii) tail meat. J. Food Sci. 53: 1666.

Vithayathill, A.J., Buck, F., Bier, M. and Nord, F.F. 1961. On the mechanism of enzyme action, LXXII. Comparative studies of trypsin from various origins. Arch. Biochem. Biophys. 92: 532.

Vitzthym, O.G. and Werkhoff, P. 1978. Aroma analysis of coffee, tea, and cocoa by headspace techniques. In "Analysis of Foods and Beverages. Headspace Techniques," G. Charalambous (Ed.). Academic Press, NY.

Vonk, H. and Western, J. 1984. "Comparative Biochemistry of Enzymatic Digestion," p. 184. Academic Press, London.

Wagner, F.W. 1988. Preparation of metal-free enzymes. In "Methods in Enzymology". J.F. Riordan and B.L. Vallee (Ed.), p. 21, Vol. 158, Academic Press. NY.

Walker, J.E. and Keil, B. 1973. Purification and characterization of different active forms of pork trypsin. Eur. J. Biochem. 32: 486.

Walradt, J.P., Pittet, A.O., Kinlin, T.E., Muralidhara, R. and Sanderson, A. 1971. Volatile components of roasted peanuts. J. Agric. Food Chem. 19: 972.

Walsh, K. and Neurath, H, 1964. Trypsinogen and chymotrypsinogen as homologous proteins. Proc. Natl. Acad. Sci. U.S. 52: 884.

Walsh, K.A. 1970. Trypsinogens and trypsins of various species. In "Methods in Enzymology." S.P. Colowick and N.O. Kaplan (Ed.), p. 41, Vol. XIX. Academic Press, NY.

Watanabe, K. and Sato, Y. 1971. Some Alkyl-substituted pyrazines and pyridines in the flavor components of shallow fried beef. J. Agric. Food Chem. 19: 1017.

Wedler, F.C. and Hoffmann, F.M. 1974. Glutamine synthetase of Bacillus stearothermophilus. I. Purification and basic properties. *Biochem.* 13: 3207.

Welgus, H.G., Grant, G.A., Jeffrey, J.J. and Eisen, A.Z. 1982. Substrate specificity of the collagenolytic serine protease from Uca pugilator: Studies with collagenous substrate. *Biochem.* 21: 5183.

Welgus, H.G. and Grant, G.A. 1983. Degradation of collagen substrates by a trypsin-like serine protease from the fiddler crab Uca pugilator. *Biochem.* 22: 2228.

Werkhoff, P., Bruning, J., Emberger, R., Guntert, M., Kopsel, M., Kuhn, W. and Surburg, H. 1990. Isolation and characterization of volatile sulfur-containing meat flavor components in model systems. *J. Agric. Food Chem.* 38: 777.

Winter, W.P. and Neurath, H. 1970. Purification and properties of a trypsin-like enzyme from the starfish Evasterias trochelii. *Biochem.* 9: 4673.

Wolz, R.L., Zeggaf, C., Stocker, W. and Zwilling, R. 1990. Thiol containing compounds and amino acid hydroxamates as reversible synthetic inhibitors of Astacus protease. *Arch. Biochem. Biophys.* 281: 275.

Yang, Y.J. and Davies, D.M. 1968. Digestion, emphasizing trypsin activity, in adult simuliids (Diptera) fed blood, blood sucrose mixtures, and sucrose. *J. Insect Physiol.* 14: 205.

Yates, L.D. and Greaser, M.L. 1983. Quantitative determination of myosin and actin in rabbit skeletal muscle. *J. Mol. Biol.* 168: 123.

Yoshinaka, R., Sato, M. and Ikeda, S. 1981. Distribution of trypsin and chymotrypsin and their zymogens in digestive system of catfish. *Bull. Jap. Soc. Sci. Fish.* 47: 1615.

Yoshinaka, R., Suzuki, T. and Sato, M. 1983. Purification and some properties of anionic trypsin from the catfish pancreas. *Bull. Jap. Soc. Sci. Fish.* 49: 207.

Yoshinaka, R., Sato, M., Tanaka, H., and Ikeda, S. 1984. Purification and characterization of a new metalloproteinase with elastolytic activity from the catfish pancreas. *Biochim. Biophys. Acta.* 798: 240.

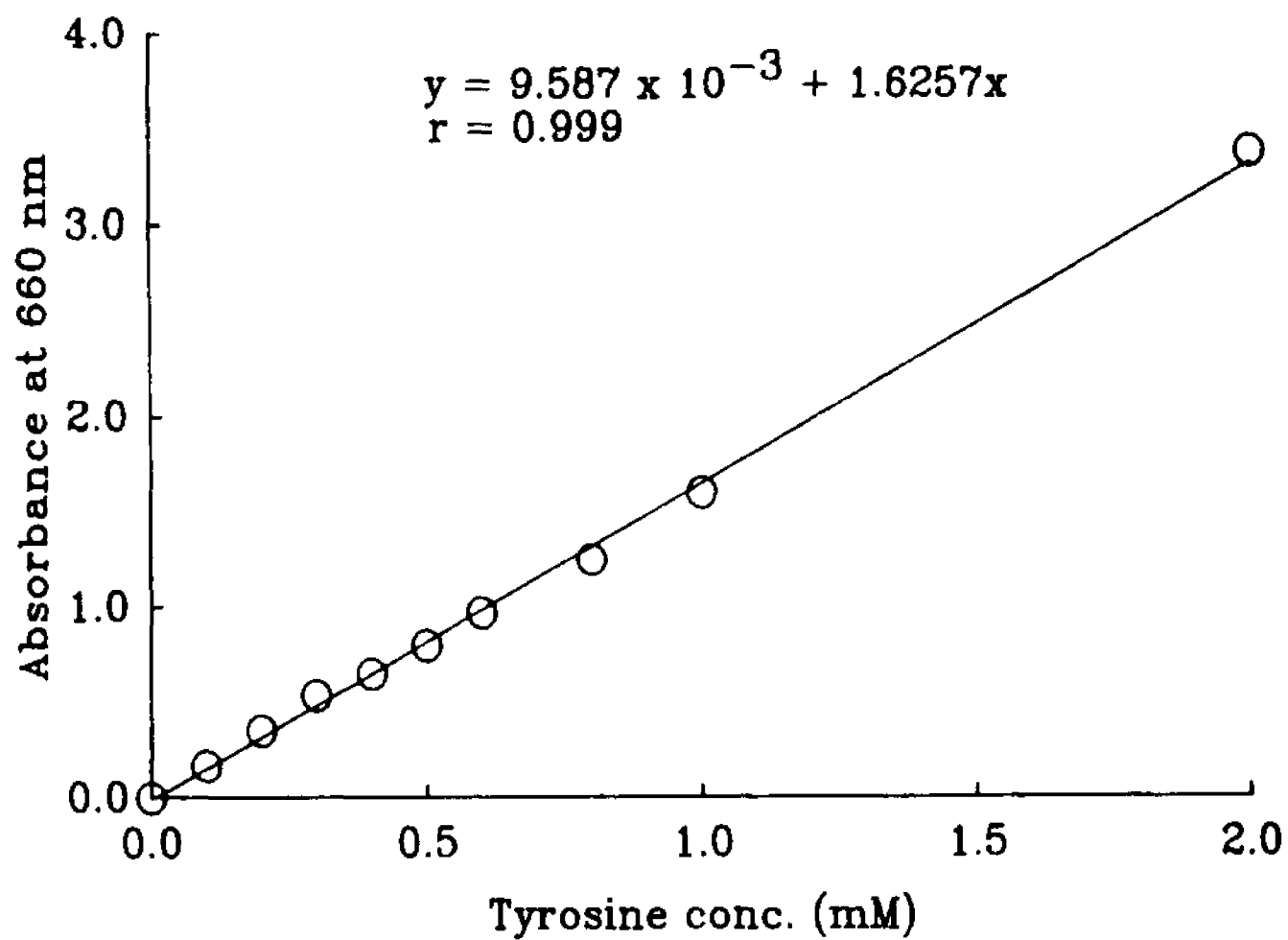
Zeece, M.G. and Katoh, K. 1989. Cathepsin D and its effects on myofibrillar proteins: A review. *J. Food Biochem.* 13: 157.

Zwilling, R., Pfleiderer, G., Sonneborn, N.-H., Kraft, V. and Stucky, I. 1969. The evolution of endopeptidases-V. Common and different traits of bovine and crayfish trypsin. Comp. Biochem. Physiol. 28: 1275.

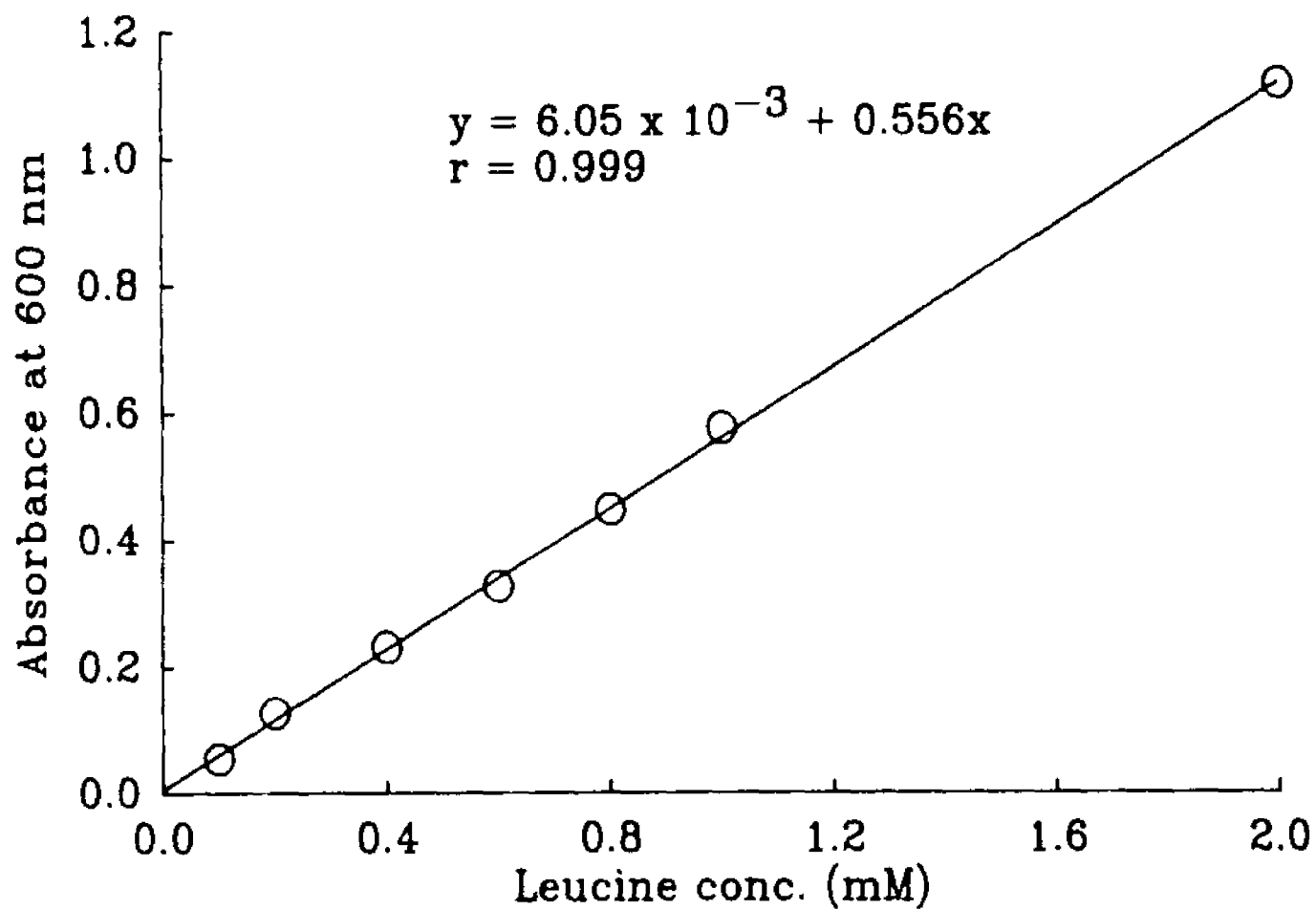
Zwilling, R. and Tomasek, V. 1970. Amino-acid composition of crayfish trypsin. Nature 228: 57.

Zwilling, R., Neurath, H., Ericsson, L.H. and Enfield, D.L. 1975. The amino-terminal sequence of an invertebrate trypsin (Crayfish) Astacus leptodactylus): homology with other serine proteases. FEBS lett. 60: 247.

Zwilling, R. and Neurath, H. 1981. Invertebrate proteases. In "Methods in Enzymology," L.Lorand (Ed.). Vol. 80, P. 633. Academic Press. NY.



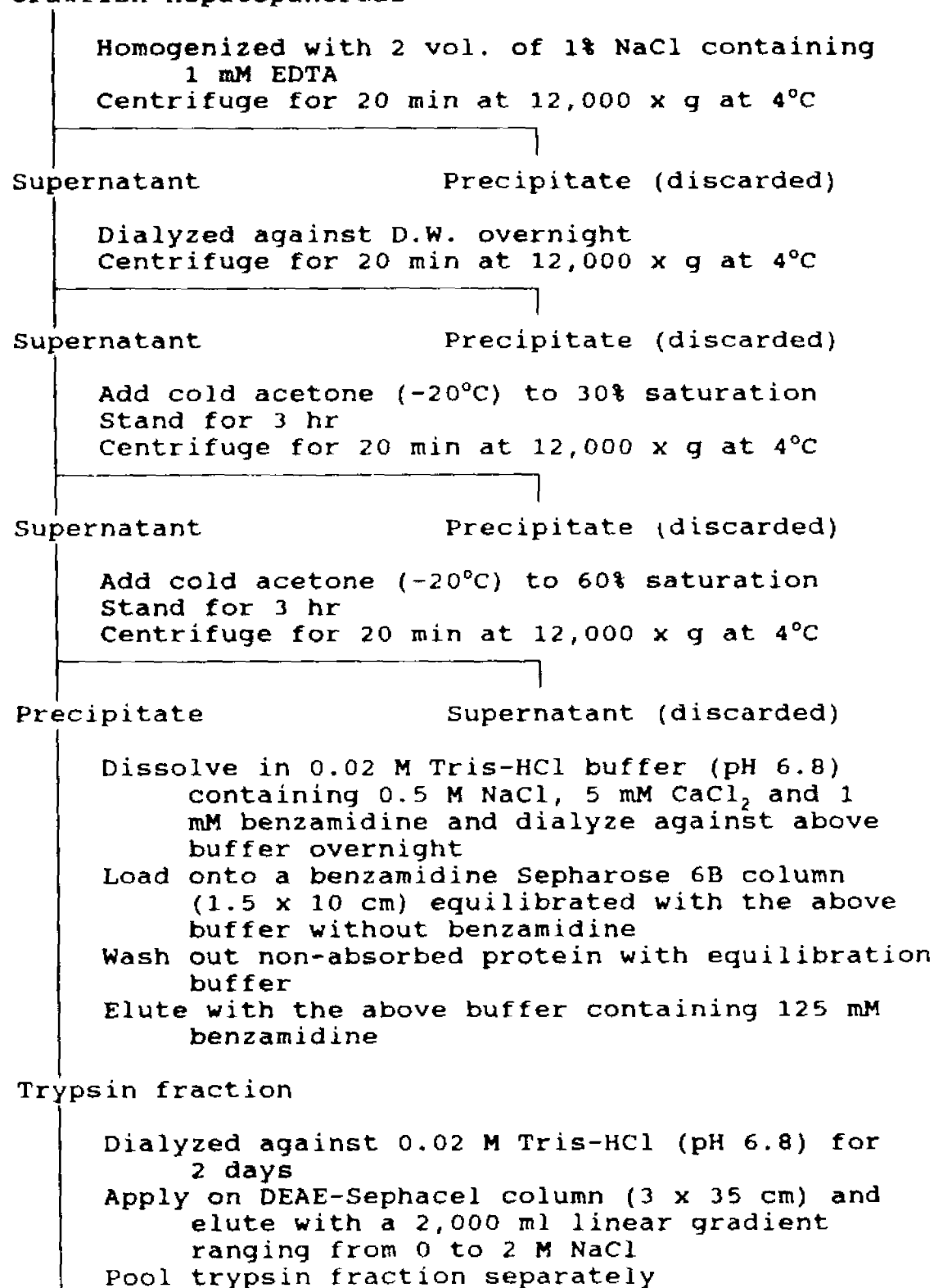
Appendix 1. Standard curve for the determination of caseinolytic activity.



Appendix 2. Standard curve for the determination of collagenolytic activity.

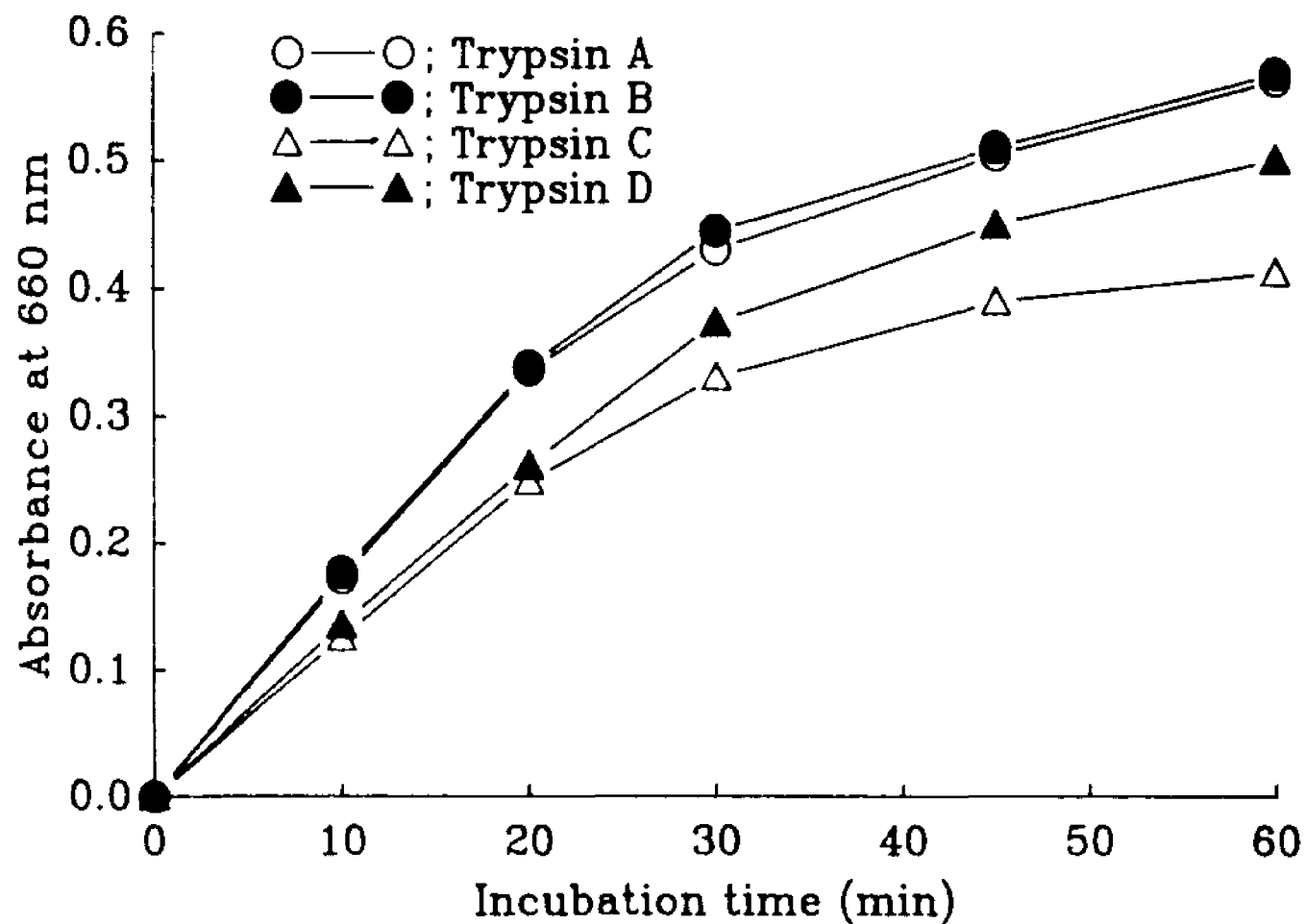
Appendix 3. Scheme of purification for anionic trypsins from the hepatopancreas of crawfish.

Crawfish hepatopancreas



Appendix 3 (continued)

Trypsin A & B		Trypsin C	Trypsin D
Apply trypsin A and B fraction on a 2'nd DEAE-Sephacel chromatographic column (2 x 20 cm) and elute with a 1,000 ml linear gradient ranging from 0.5 to 1.5 M NaCl			
Pool and concentrate with ultrafiltration			
Dialyzed against 0.02 M Tris-HCl (pH 6.8) + 0.1 M NaCl			
Trypsin A	Trypsin B	Trypsin C	Trypsin D
Apply to a Sephacryl S-200 column (2.5 x 90 cm) equilibrated with 0.02 M Tris-HCl (pH 6.8) containing 0.1 M NaCl			
Elute trypsins separately with the same buffer			
Concentrate with acetone precipitation			
Dissolve in D.W.			
Dialyzed against D.W. overnight and stored at -20°C			
Purified Trypsins			



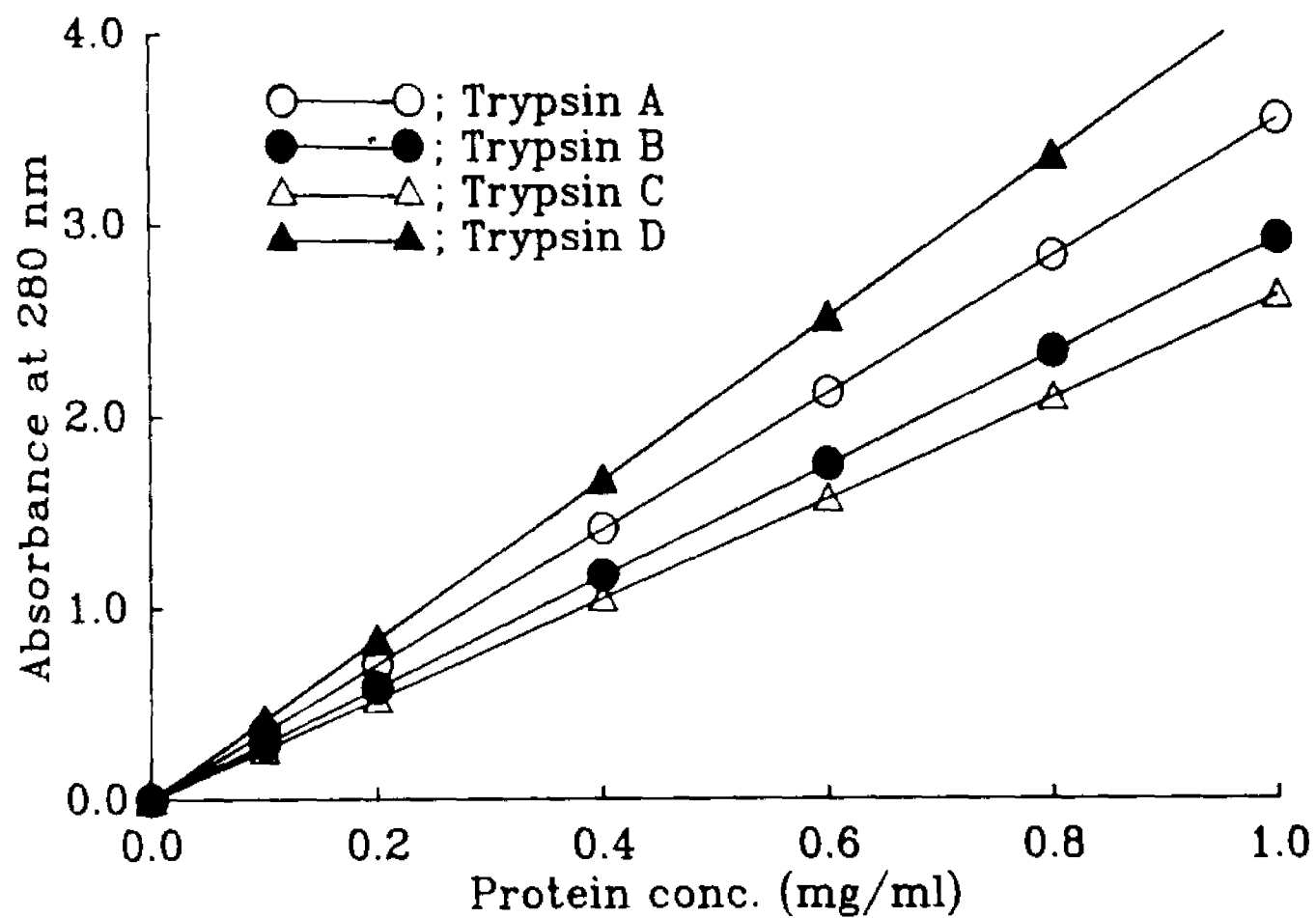
Appendix 4. Effect of reaction time on the hydrolysis of casein by purified trypsins from crawfish hepatopancreas.

Appendix 5. Characteristic ion m/z
(mass/charge) values

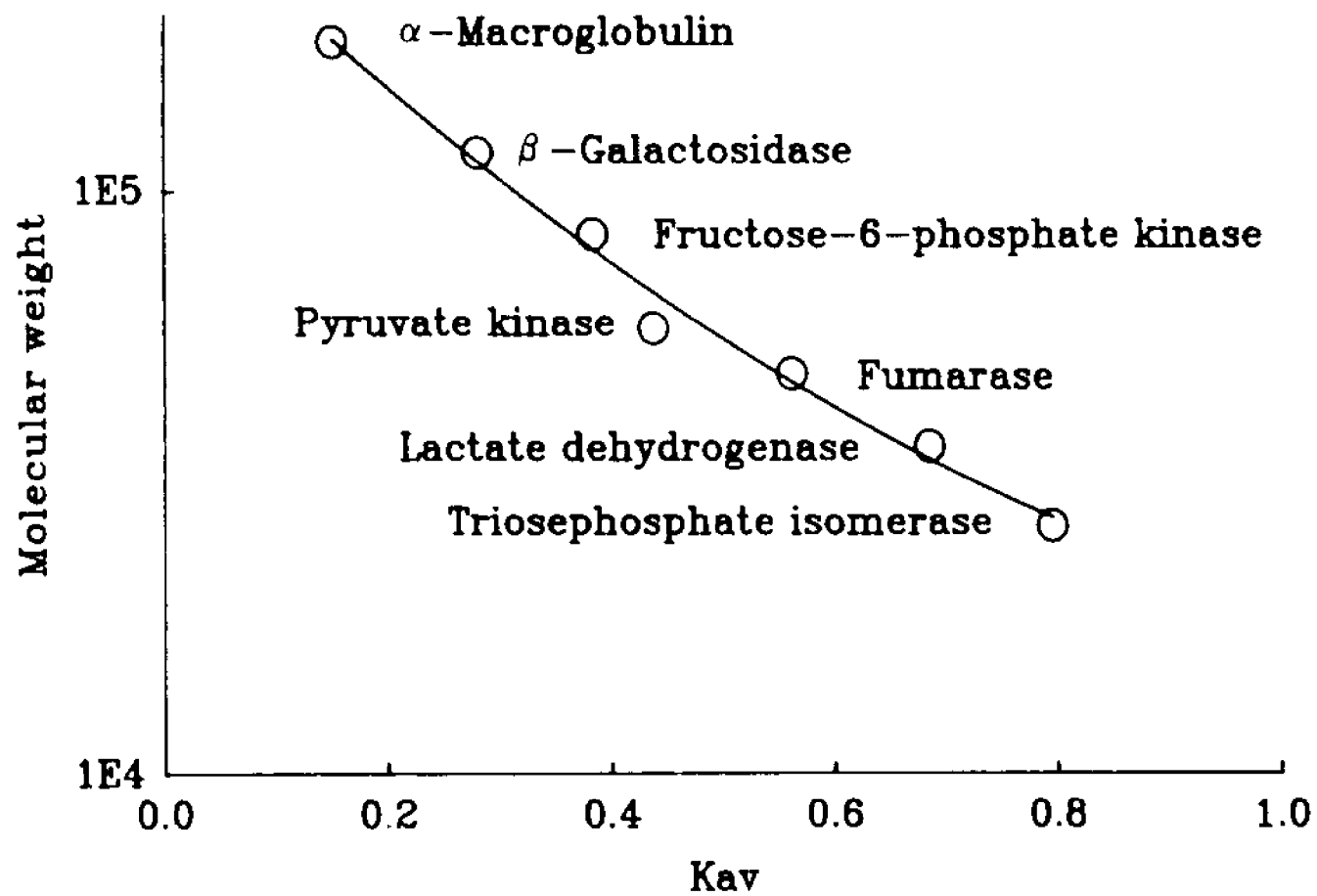
Compounds	Ion m/z value
Haptanal	83
Nonanal	57
(E,E)-2,4-Hexadienal	81
(E,E)-2,4-Heptadienal	81
(E)-2-Nonenal	70
(E,Z)-2,6-Nonadienal	70
(E,E)-2,4-Octadienal	81
Butanol	45
(E)-2-Penten-1-ol	57
Hexanol	56
Octanol	56
2-Methoxyphenol	109
2-Furanmethanol	98
Benzenemethanol	108
Benzene	78
Toluene	91
Ethylbenzene	91
2-Ethyltoluene	105
1,2,4,5-Tetramethylbenzene	119
1,4-Dichlorobenzene	146
Dimethylphenol	107
2,4-Dimethylcyclobutanone	56
2-Decanone	58
Undecanone	58
1-Phenylethanone	105
Tridecanone	58
Tetradecanone	58
Pentadecanone	58
Methylpyrazine	94
2,5-Dimethylpyrazine	108
2,6-Dimethylpyrazine	108
Ethylpyrazine	107
2,3-Dimethylpyrazine	108
2-Ethyl-6-methylpyrazine	121
2-Ethyl-5-methylpyrazine	121
Trimethylpyrazine	122
2-Ethyl-3,6-dimethylpyrazine	135
2-Ethyl-3,5-dimethylpyrazine	135
Tetramethylpyrazine	36
Methylpyrrole	80
Dipropyl disulfide	150
2-Octene	55
2-Methylfuran	82
2-Ethylfuran	81
Chloroform	83

Appendix 5 (continued)

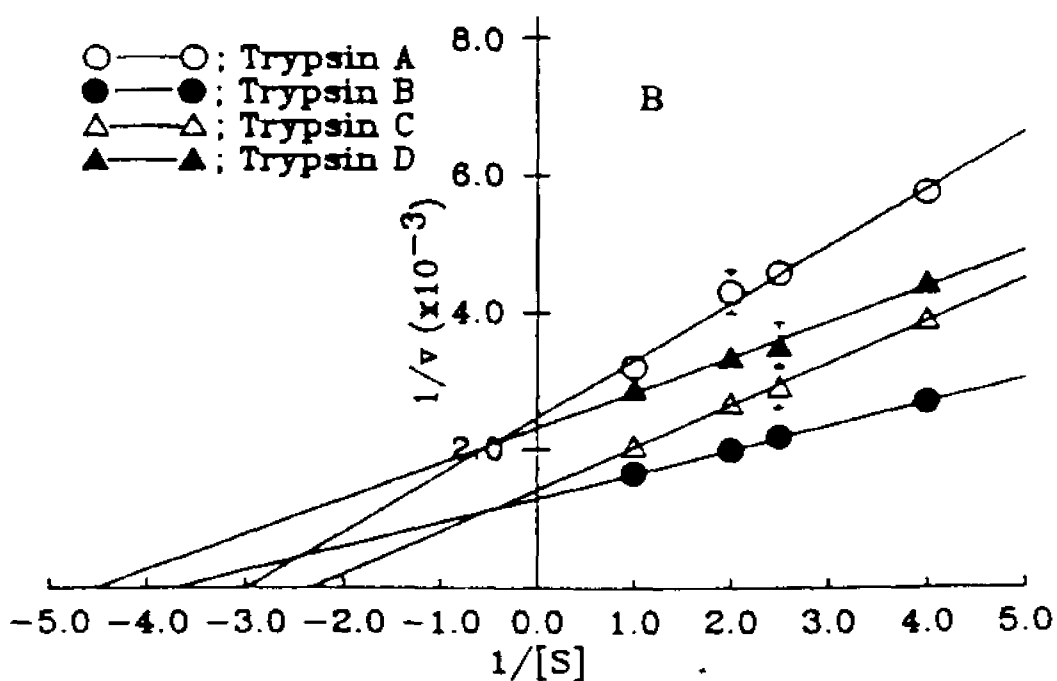
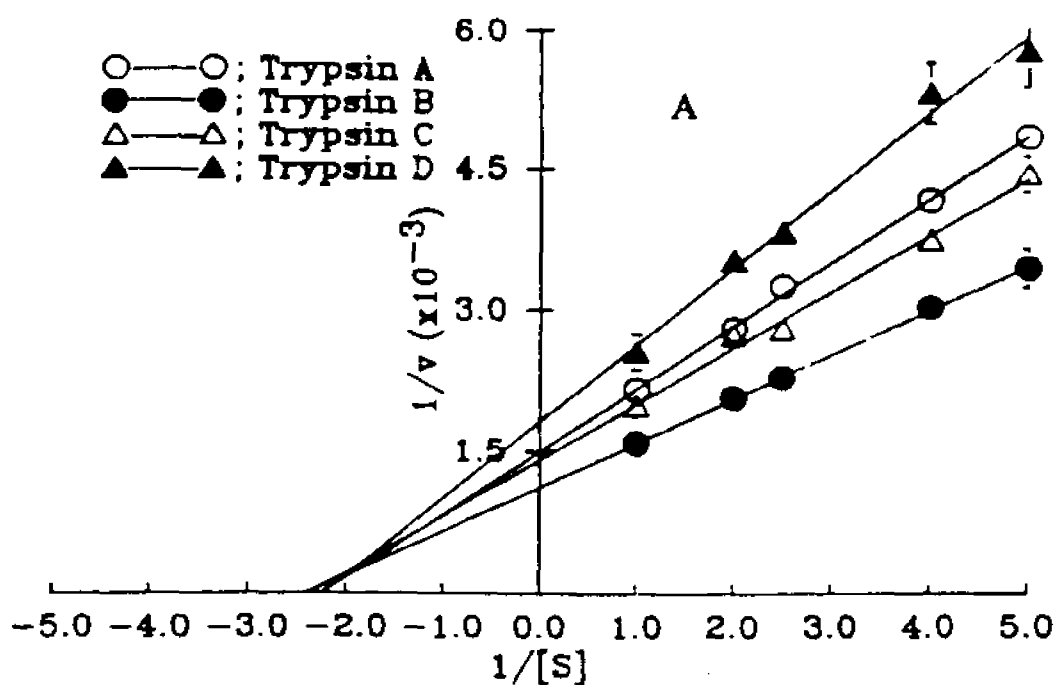
Dodecane	58
Pentylfuran	81
Octylfuran	81
α -Terpineol	136
Heptadecane	57
Naphthalene	128
Hexanoic acid	60
Methylnaphthalene	141
Benzeneacetonitrile	117
β -Ionone	177



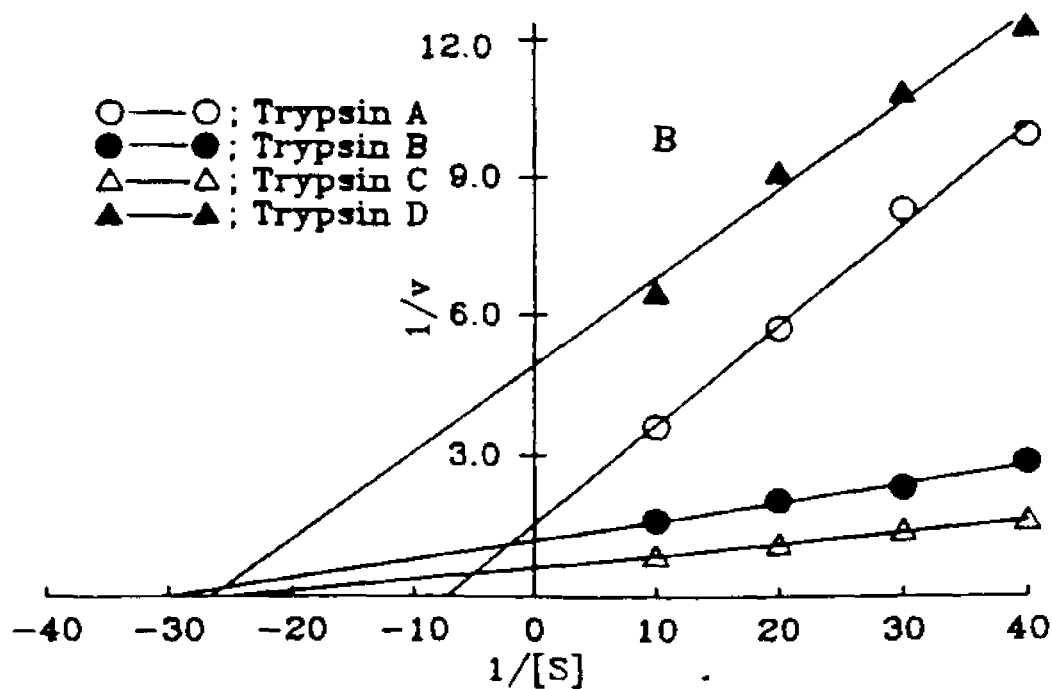
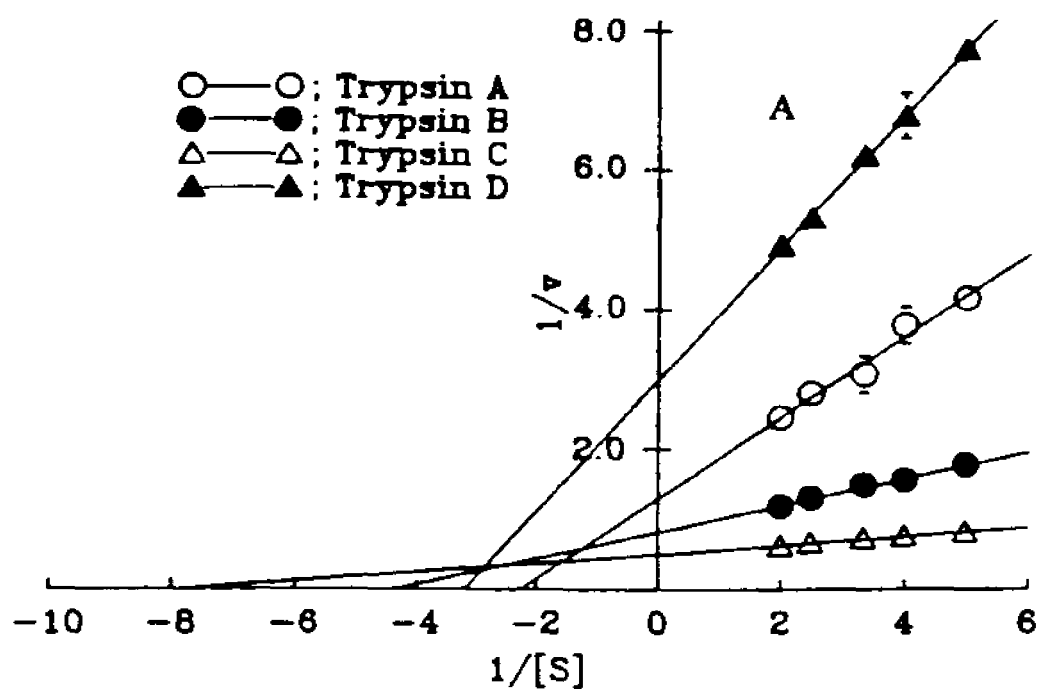
Appendix 6. UV absorbance of purified trypsins from crawfish hepatopancreas at 280 nm.



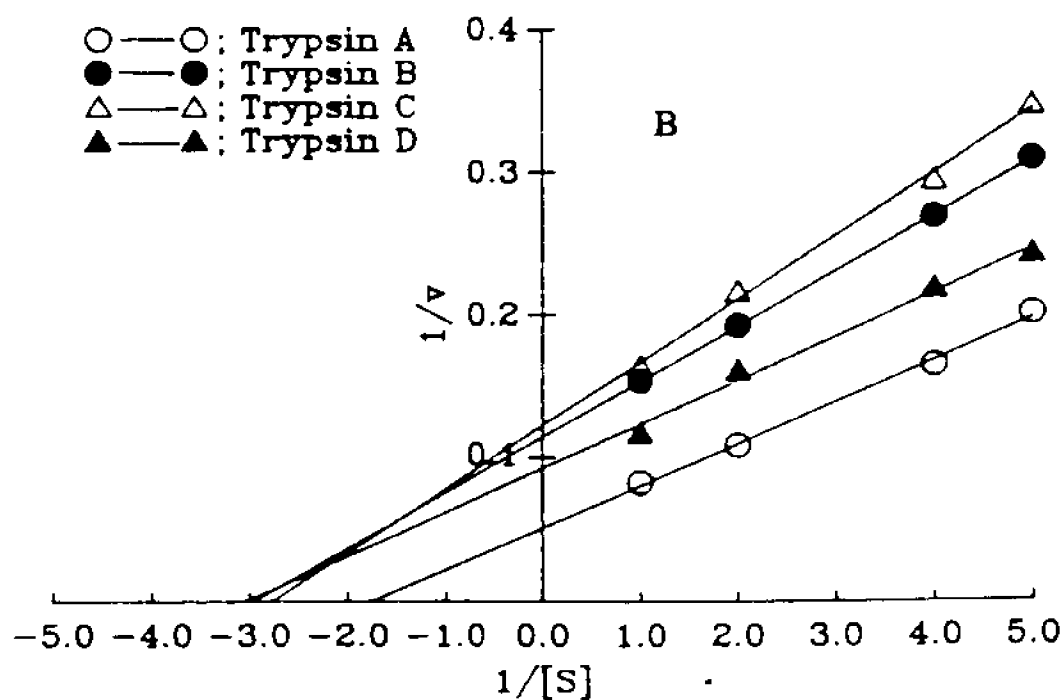
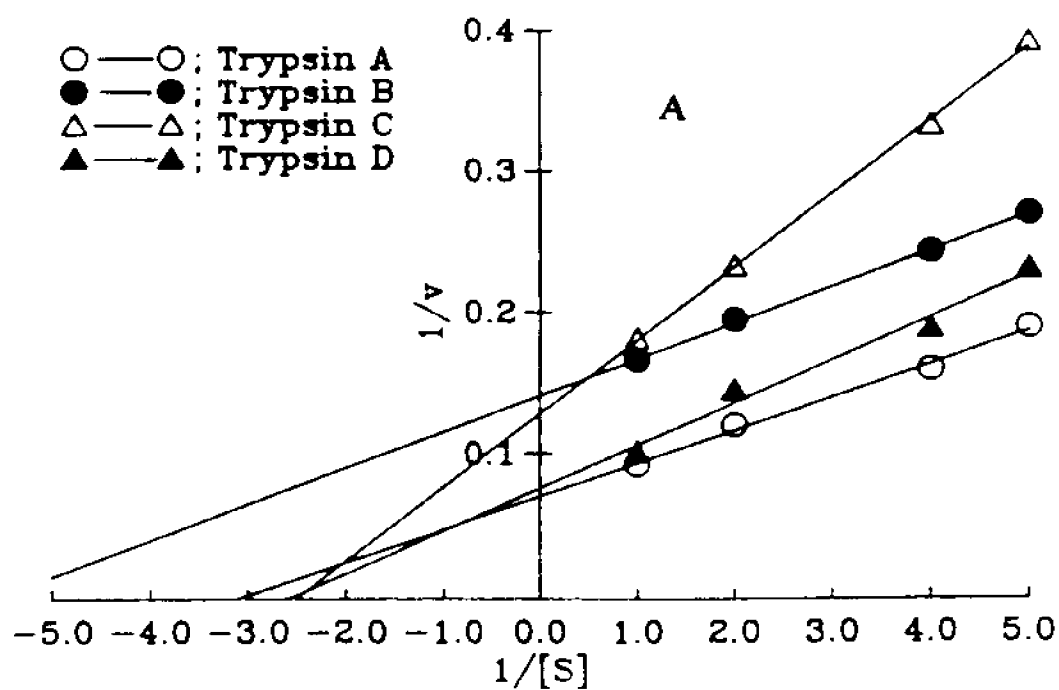
Appendix 7. Calibration curve obtained with proteins from the prestained SDS molecular weight standard mixture (SDS-7B, Sigma, MO)



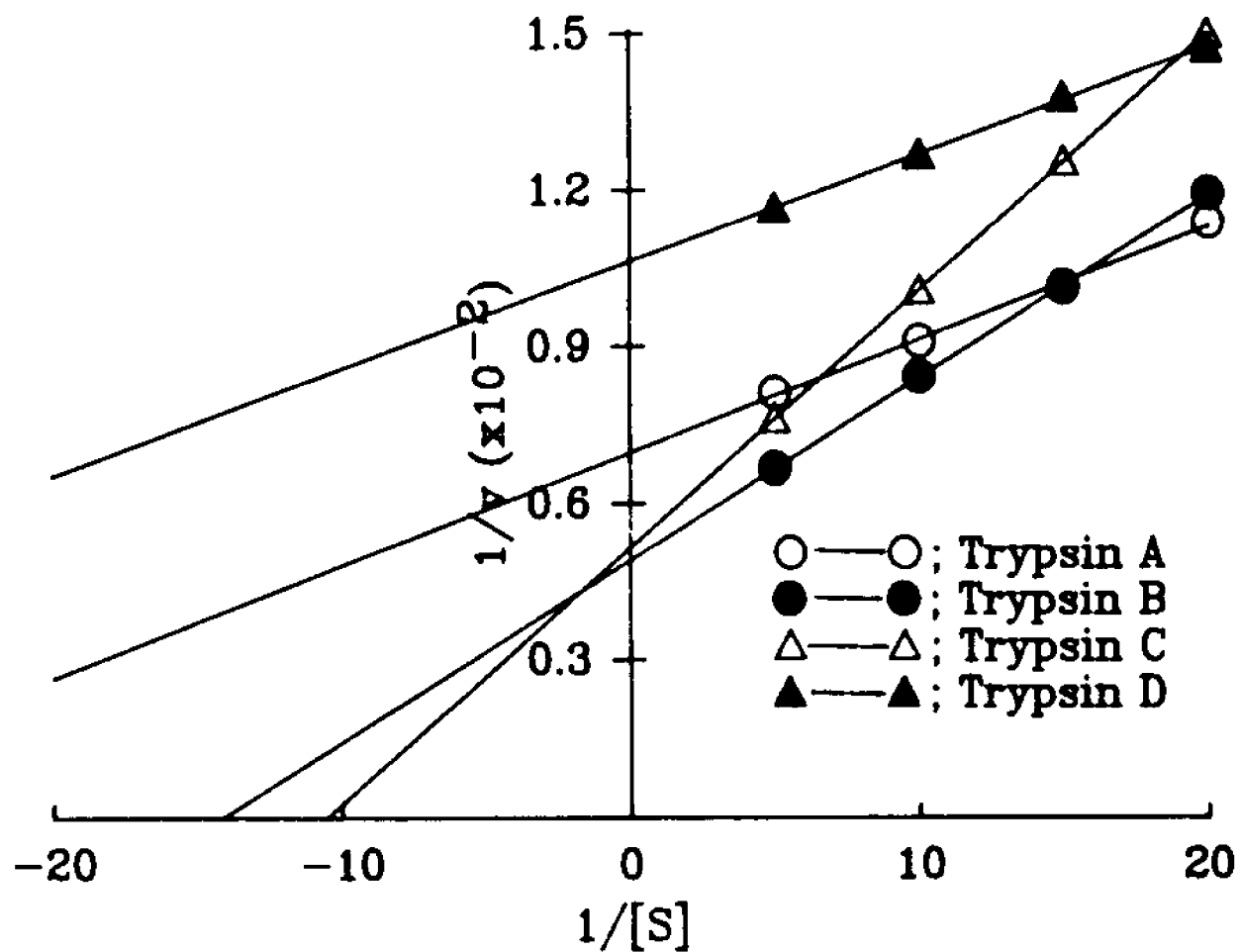
Appendix 8. Lineweaver-Burk plots for hydrolysis of p-tosyl-L-arginine methyl ester (TAME) at pH 6.8 (A) and 8.1 (B) by purified trypsins from crawfish hepatopancreas.



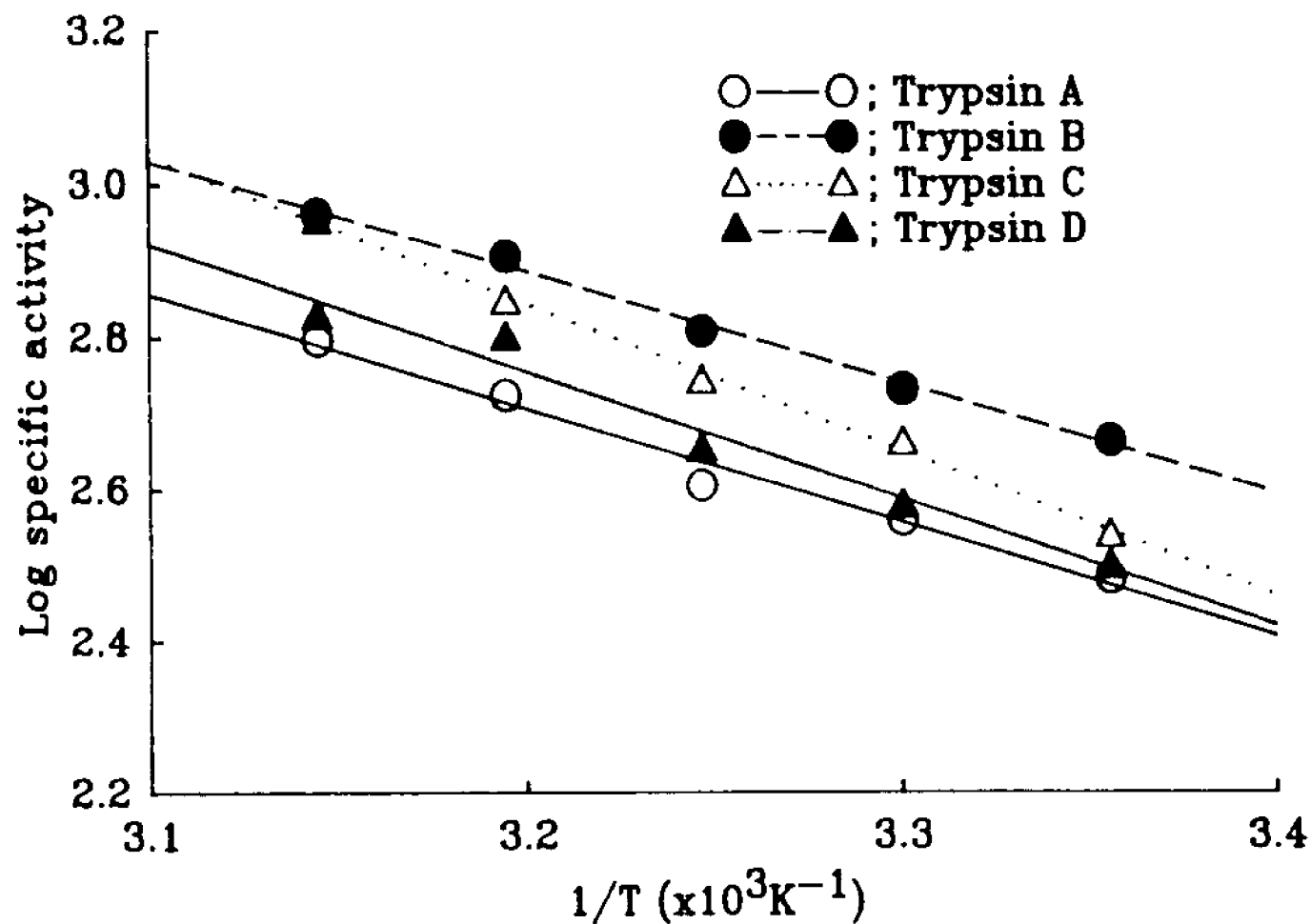
Appendix 9. Lineweaver-Burk plots for the hydrolysis of benzoyl-D,L-arginine-p-nitroanilide (BAPNA) at pH 6.8 (A) and 8.1 (B) by purified trypsins from crawfish hepatopancreas.



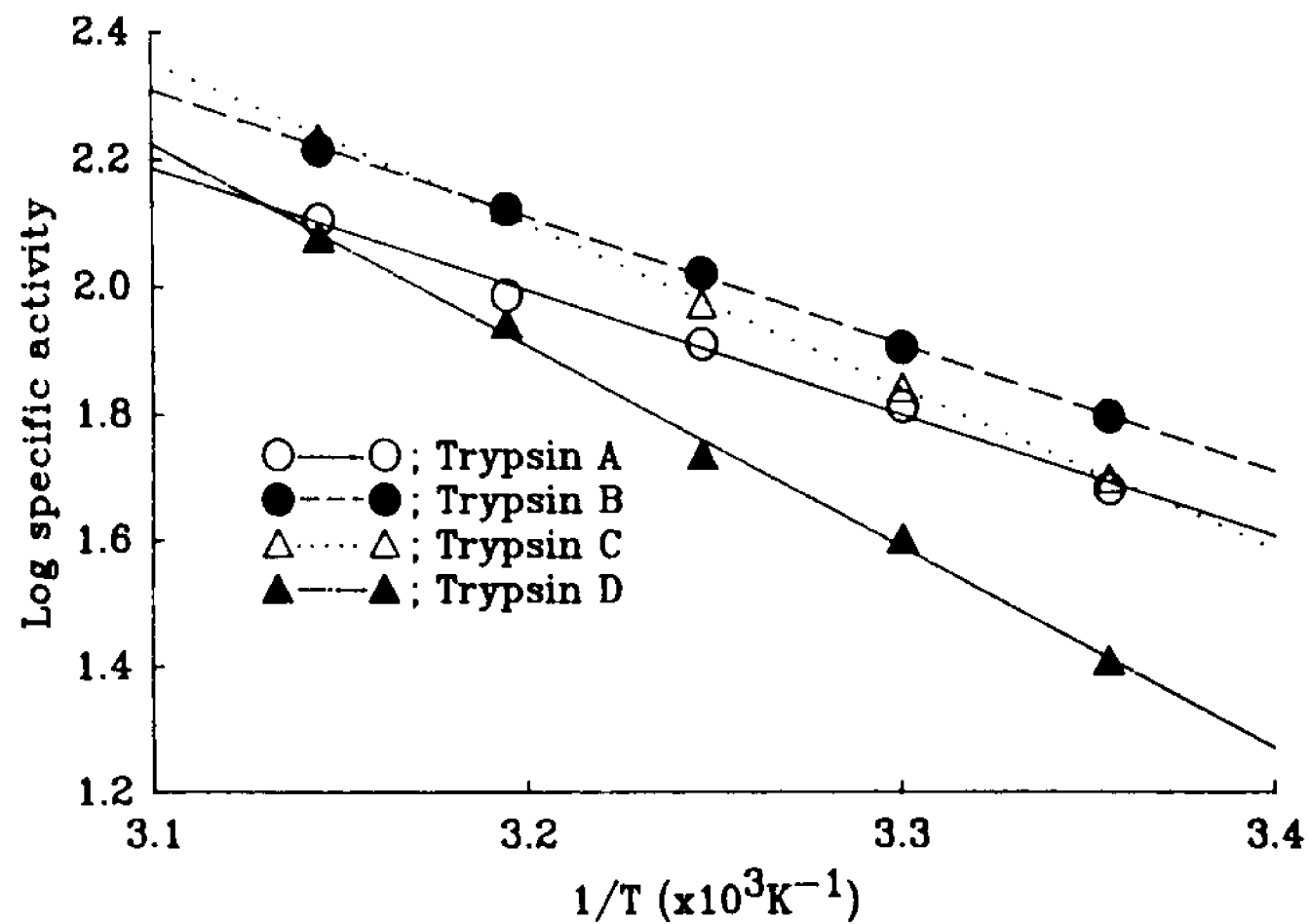
Appendix 10. Lineweaver-Burk plots for hydrolysis of casein at pH 6.8 (A) and 8.1 (B) by purified trypsins from crawfish hepatopancreas.



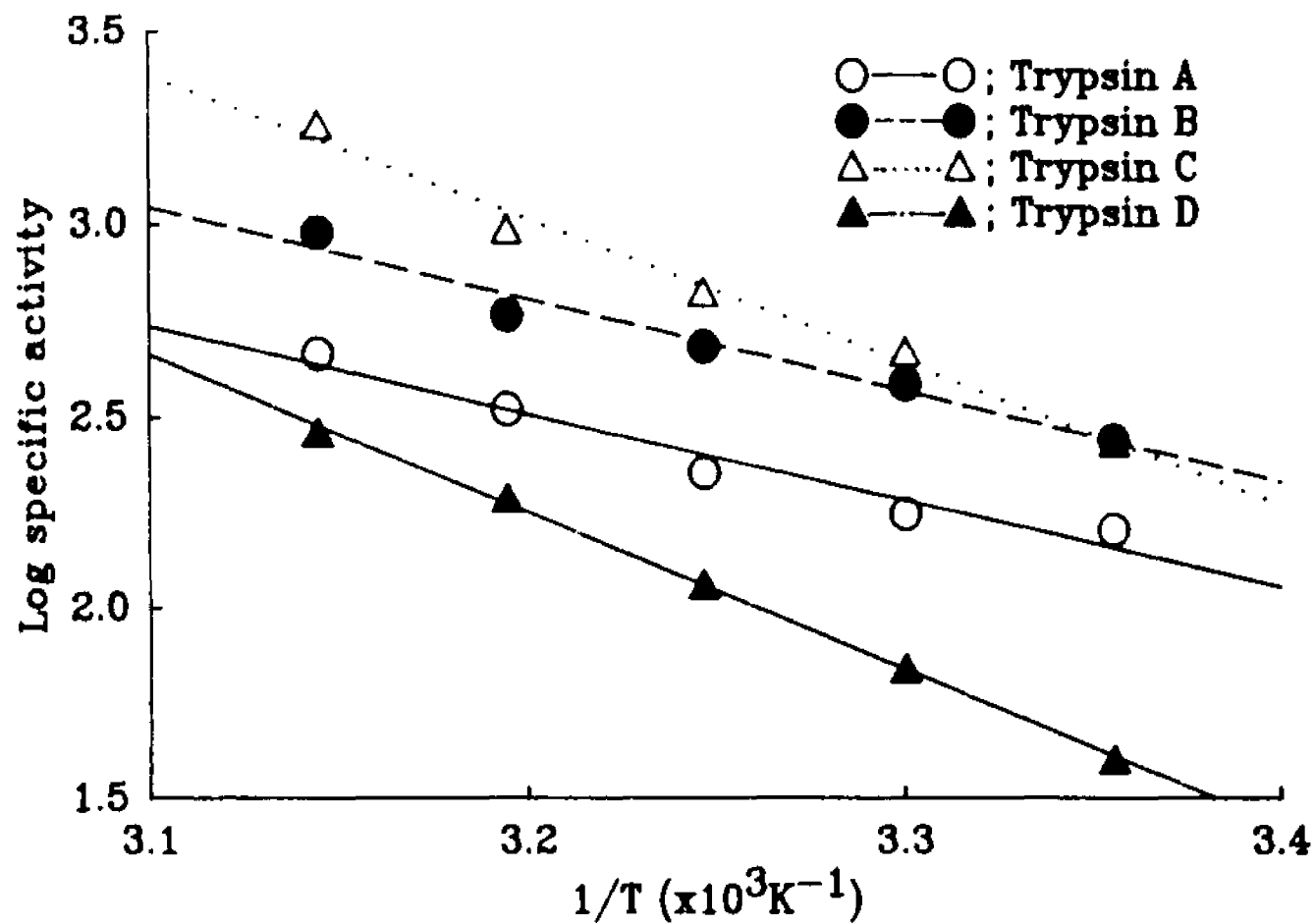
Appendix 11. Lineweaver-Burk plots for hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) at pH 8.1 by purified trypsins from crawfish hepatopancreas.



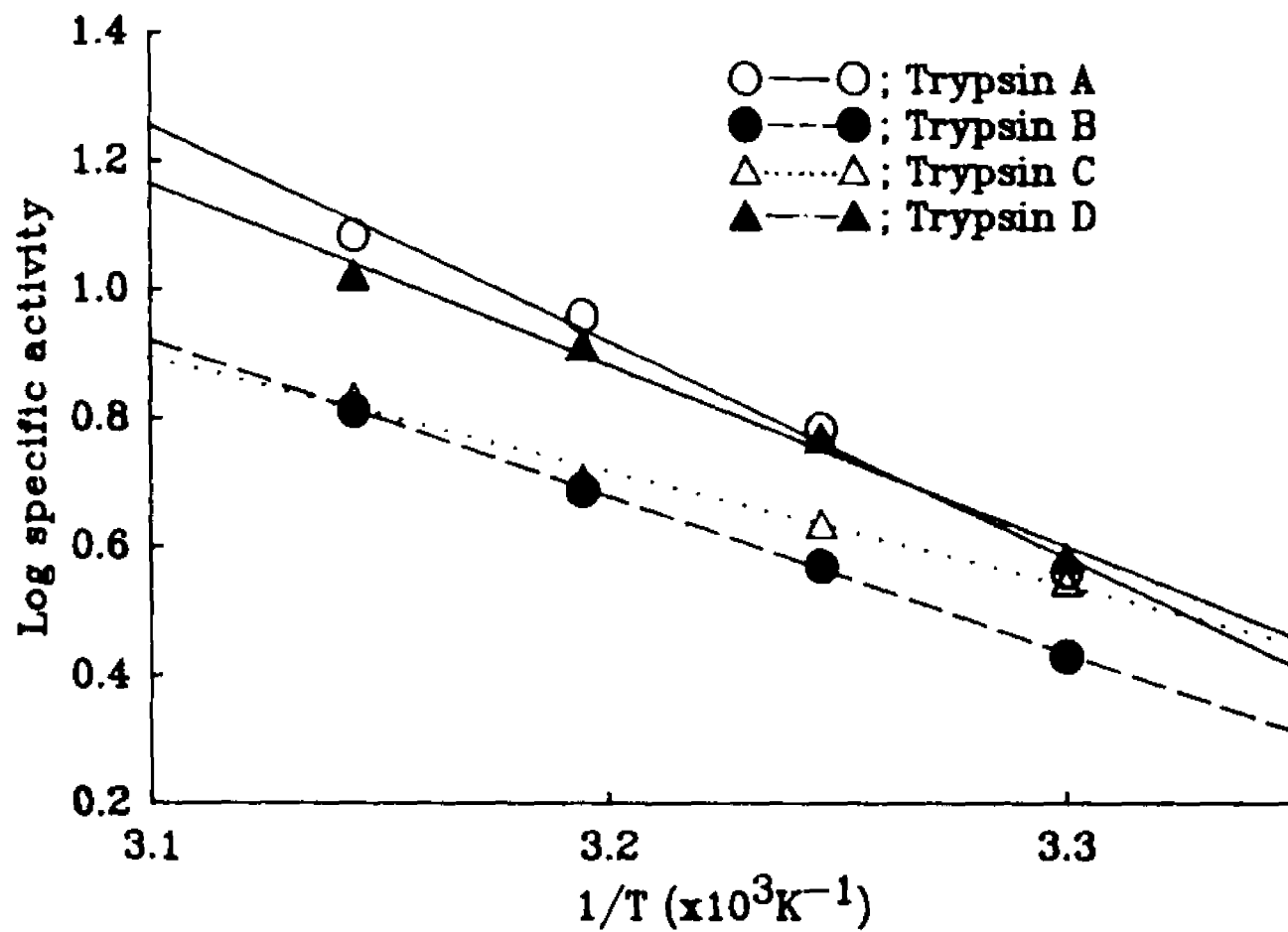
Appendix 12. Arrhenius plots for hydrolysis of p-tosyl-L-arginine methyl ester (TAME) at pH 6.8 by crawfish hepatopancreatic trypsins.



Appendix 13. Arrhenius plots for hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) at pH 6.8 by crawfish hepatopancreatic trypsins.



Appendix 14. Arrhenius plots for hydrolysis of benzoyl-D,L-arginine-p-nitroanilide (BAPNA) at pH 6.8 by crawfish hepatopancreatic trypsins.



Appendix 15. Arrhenius plots for hydrolysis of casein at pH 6.8 by crawfish hepatopancreatic trypsins.

VITA

The author was born in Kyeong-Nam, Korea, on January 12, 1957. He entered National Fisheries University of Pusan in 1977. After his freshman year, he served in the army for 3 years as step-sergent and was discharged in August, 1980. In February, 1984, he graduated from National Fisheries University of Pusan with a Bachelor of Engineering degree in Food Science and Technology. After graduation, he entered the Graduate School in Food Science and Technology of National Fisheries University of Pusan on March, 1984 and studied the Purification and Characterization of Fish Proteases. He graduated from National Fisheries University of Pusan with a Master of Engineering degree in Food Science and Technology in 1986. During his Master course he worked as a research assistant in the Department of Nutrition and Food Science, and the assistantship was continued until August, 1987 at which time he entered the Food Science Department, Louisiana State University where he is currently a candidate for the degree of Doctor of Philosophy.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate Hyeung-Rak Kim

Major Field: Food Science

Title of Dissertation CHARACTERIZATION AND POTENTIAL UTILIZATION OF PROTEASES
FROM THE HEPATOPANCREAS OF CRAWFISH, Procambarus clarkii

Approved

J. Samuel Godber
Major Professor and Chairman

H. Kim

Dean of the Graduate School

EXAMINING COMMITTEE

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Date of Examination

June 14, 1991